The genome packaging machinery of dsDNA bacteriophage PRD1

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ACADEMIC DISSERTATION

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”Tämän luulen oppineeni: todellisuus on vain työhypoteesi. Se on sopimus, jota emme tiedä tehneemme. Se on harha, jonka jokainen näkee. Mutta se on yhteinen, välttämätön illuusio, se on terveen järkemme, mielikuvituksemme ja aistiemme lopputuote, terveytemme ja työkykymme perusta, meidän totuumme.”

(Leena Krohn, Datura - *Tai harha jonka jokainen näkee*. WSOY 2001).

“Oh but it's like this, look you, I've no reason to be discontented with my polyhedra; they bear their young every six weeks, it's worse than rabbits. And it's also quite true to say that the regular polyhedra are the most faithful and devoted to their master, except that this morning the Icosahedron was a little fractious, so that I was compelled, look you, to give it a slap on each one of its faces. And that's the sort of language they understand.”

(professor Achras in Act I of Ubu Cucu (Ubu Cuckolded), by Alfred Jarry, from a version by Cyril Connolly)

Original publications

This thesis is based on the following articles, which are referred to in the text by the respective Roman numerals

I Strömsten N.J., Bamford D.H. and Bamford J.K.H. 2003. The unique vertex of bacterial virus PRD1 is connected to the viral internal membrane. *J. Virol.* 77(11):6314-21.

II Strömsten N.J., Benson S.D., Burnett R.M., Bamford D.H. and Bamford J.K.H. 2003. The *Bacillus thuringiensis* linear double-stranded DNA phage Bam35, which is highly similar to the *Bacillus cereus* linear plasmid pBClin15, has a prophage state. *J. Bacteriol.* 185(23):6985-9.

III Strömsten N.J., Bamford D.H.and Bamford J.K.H. 2005. *In vitro* DNA packaging of PRD1: a common mechanism for internal-membrane viruses. *J. Mol. Biol.* 348(3):617-29.

IV Karhu, N.J., Ziedaite, G., Bamford, D.H. and Bamford, J.K.H. Efficient DNA packaging of bacteriophage PRD1 requires the unique vertex protein P6. Submitted.

Abbreviations

aa	amino acid
AFM	atomic force microscopy
Amp, Ap	ampicillin
ATP	adenosine triphosphate
bp	base pair
C-	carboxy (when in polypeptide)
cfu	colony-forming units
CM	cytoplasmic membrane
Cm	chloramphenicol
CTP	cytidine triphosphate
Da	dalton
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
DNA	deoxyribonucleic acid
ds	double-stranded
dU	deoxyuridine
EM	electron microscopy
gp	gene product
GTP	guanosine triphosphate
ICTV	International Committee on the Taxonomy of Viruses
IHF	*E. coli* integration host factor
Inc	incompatibility
IPTG	isopropyl-β-D-thiogalactopyranoside
ITR	inverted terminal repeat
IV	immature virion
kb, kbp	kilobase pairs
kDa	kilodalton
LPS	lipopolysaccharide
LUCA	last universal common ancestor
LIN	lysis inhibition
Mpf	Mating pair formation
N-	amino (when in polypeptide)
NAG	N- acetylglucosamine
NAM	N- acetylmuramic acid
NCLDV	nucleo-cytoplasmic large DNA virus
nt	nucleotide
NTP	nucleoside triphosphate
OM	outer membrane
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAL	peptidoglycan-associated lipoprotein
PE	phosphatidyl ethanolamine
PEG	polyethylene glycol
pfu	plaque-forming units
PG	phosphatidyl glycerol
pI	iso-electric point
PCR	polymerase chain reaction
PMF	proton-motive force
pRNA	*p*rohead RNA or *p*ackaging RNA
R	rough (for LPS type)
RNA	ribonucleic acid
S	smooth (for LPS type)
SDS	sodium dodecyl sulphate
ss	single-stranded
sus	suppressor sensitive
T-number	triangulation number
TEM	transmission electron microscopy
UTP	uridine triphosphate
VLP	virus-like particle
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abbreviations of virus names

ABV	Acidianus bottle-shaped virus
ASFV-1	African swine fever virus
ATV	Acidianus two-tailed virus
Bam35c	clear plaque mutant of Bam35
CIV	Chilo iridescent virus
ESV-1	Ectocarpus siliculosis virus 1
HIV-1	Human immunodeficiency virus 1
HRV14	Human rhinovirus 14
HSV-1	Herpes simplex virus 1
PBCV-1	Paramecium bursaria Chlorella virus 1
PpV01	Phaeocystis pouchetii virus
SNDV	Sulfolobus neozealandicus droplet-shaped virus
SSV1	Sulfolobus spindle-shaped virus 1
STIV	Sulfolobus turreted icosahedral virus
STSV-1	Sulfolobus tengchongensis spindle-shaped virus 1
TMV	Tobacco mosaic virus
VV	Vaccinia virus

Summary

Viral genomes are encapsidated within protective protein shells. This encapsidation can be achieved either by a co-condensation reaction of the nucleic acid and coat proteins, or by first forming empty viral particles which are subsequently packaged with nucleic acid, the latter mechanism being typical for many dsDNA bacteriophages. Bacteriophage PRD1 is an icosahedral, non-tailed dsDNA virus that has an internal lipid membrane, the hallmark of the *Tectiviridae* family. Although PRD1 has been known to assemble empty particles into which the genome is subsequently packaged, the mechanism for this has been unknown, and there has been no evidence for a separate packaging vertex, similar to the portal structures used for packaging in the tailed bacteriophages and herpesviruses.

In this study, a unique DNA packaging vertex was identified for PRD1, containing the packaging ATPase P9, packaging factor P6 and two small membrane proteins, P20 and P22, extending the packaging vertex to the internal membrane. Lack of small membrane protein P20 was shown to totally abolish packaging, making it an essential part of the PRD1 packaging mechanism. The minor capsid proteins P6 was shown to be an important packaging factor, its absence leading to greatly reduced packaging efficiency. An *in vitro* DNA packaging mechanism consisting of recombinant packaging ATPase P9, empty procapsids and mutant PRD1 DNA with a *LacZ*α-insert was developed for the analysis of PRD1 packaging, the first such system ever for a virus containing an internal membrane.

A new tectiviral sequence, a linear plasmid called pBClin15, was identified in *Bacillus cereus*, providing material for sequence analysis of the tectiviruses. Analysis of PRD1 P9 and other putative tectiviral ATPase sequences revealed several conserved sequence motifs, among them a new tectiviral packaging ATPase motif. Mutagenesis studies on PRD1 P9 were used to confirm the significance of the motifs. P9-type putative ATPase sequences carrying a similar sequence motif were identified in several other membrane containing dsDNA viruses of bacterial, archaeal and eukaryotic hosts, suggesting that these viruses may have similar packaging mechanisms. Interestingly, almost the same set of viruses that were found to have similar putative packaging ATPases had earlier been found to share similar coat protein folds and capsid structures, and a common origin for these viruses had been suggested. The finding in this study of similar packaging proteins further supports the idea that these viruses are descendants of a common ancestor.

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1 INTRODUCTION

Even though viruses are often considered only for their pathogenic potential in humans, animals or crop plants, their role in the biosphere is far more complex and important. Viruses are the most abundant group of organisms roaming our planet and they infect cells of all kinds: plants, animals, yeasts and other organisms of the Eukarya, Bacteria and Archaea. Bacteriophages are particularly widely spread and have been found everywhere, from familiar environments such as oceans (Espejo and Canelo, 1968), sewage (Olsen et al., 1974) and agricultural and forest soil samples (Williamson et al., 2005), to such diverse locations as the Sahara desert (Prigent et al., 2005), industrial sauerkraut fermentations (Barrangou et al., 2002; Yoon et al., 2002), arctic sea ice (Borriss et al., 2003) and hot springs (Breitbart et al., 2004; Yu et al., 2006). Bacteriophages have been measured to be present in aquatic environments at numbers ranging from 10^5 plaque forming units (pfu)/ml to approximately 10^8 pfu/ml, the average in most studies being 10^7 pfu/ml (Bergh et al., 1989; Paul et al., 1991; Wommack et al., 1992; Peduzzi and Schiemer, 2004), and up to approximately 4×10^9 pfu/g (dry weight) in terrestrial soils (Williamson et al., 2005). Viruses are usually present at numbers of at least an order of a magnitude greater than their host bacteria (Bergh et al., 1989; Wommack et al., 1992; Peduzzi and Schiemer, 2004), suggesting a far more important role than previously suspected for viruses in controlling the numbers and distribution of their host species and maintaining the ecological balance of various terrestrial and aquatic environments.

Whether viruses can actually be defined as living organisms is however debatable. Viruses are obligate intracellular parasites, unable to replicate outside their host cells, and totally dependent on their host cells to provide them with both energy and building blocks for the synthesis of new virions, and the translation machinery for protein synthesis. Many viruses also use the transcription machinery and in some cases, also the DNA replication system of their host cells. However, several viruses have been found to have genes involved in processes previously thought to be performed only by proper cellular organisms: such as photosynthesis (Lindell et al., 2005; Sullivan et al., 2006), polyamine, chitin and hyaluronan biosynthesis (DeAngelis et al., 1997; Kawasaki et al., 2002; Morehead et al., 2002), nucleotide precursor biosynthesis (Landstein et al., 1996), and even protein-translation components, such as amino-acyl transfer RNA synthetases and tRNAs (Simpson et al., 2003; Raoult et al., 2004). The discovery of the giant acantamoeba polyphaga mimivirus, bigger in both virion diameter and genome length than some small bacteria (Raoult et al., 2004), has further complicated the question of what features define a cell or a virus, and stimulated the imagination of virologists and evolutionary biologists.

Grossly simplified, viruses are only genetic material, i.e. nucleic acid, wrapped within a protective protein shell. The main differences between groups of viruses arise from the type of nucleic acid, DNA or RNA, single- or double-stranded, and the shape of the protein packaging, which can be roughly spherical, filamentous or amorphous. Some viruses may have a lipid membrane inside or outside the capsid, or have other fancy accessories such as sugars, lipids or other modifications in their protein shells. However, the basic principle remains the same.

In the following chapters, the main aspects of virus structure and the virus/bacteriophage life cycle shall be described, giving special attention to virus capsid maturation and genome packaging mechanisms. Focus shall be mainly on icosahedral dsDNA viruses, and especially dsDNA bacteriophages. Additionally, virus evolution, and especially what structural analysis of viral proteins and whole virions has added to our knowledge of viral evolution, will be discussed.

1.1 Viral genomes

One way to group viruses is according to the type of nucleic acid they contain and the method of replication used. Whereas the genomes of cellular organisms always consist of double-stranded (ds) DNA, viral genomes may consist of either DNA or RNA, in single-stranded (ss) or double-stranded form, and the nucleic acid molecules may be either linear or circular. Some dsDNA viruses with partially single-stranded regions have also been detected, and in the case of RNA viruses, genomes may be divided into multiple segments.

Based on their nucleic acid content, viruses can be divided into 7 main groups, based on the suggestion by David Baltimore (1971), as follows [Virus nomenclature and division into orders, families and genera as presented in the VIIIth ICTV report (Fauquet et al., 2005)]:

I Double-stranded DNA viruses, which include for example the families *Adenoviridae* and *Herpesviridae*, and the tailed bacteriophages (order *Caudovirales*).

II Single-stranded (+)sense DNA viruses, such as the viruses of the *Parvoviridae* and *Circoviridae* families. In ssDNA viruses the single DNA strand must first be copied to dsDNA in order to allow transcription and further DNA synthesis.

III Double-stranded RNA viruses. These include for example the *Reoviridae*, *Birnaviridae* and *Cystoviridae* families. Most of the dsRNA viruses have segmented genomes, for example the cystoviruses have three genome segments and the reoviruses 10-12 segments, but viruses of the *Toti*- and *Hypoviridae* have only one segment.

IV Single-stranded (+)sense RNA viruses. In (+)sense ssRNA viruses the genome can directly serve as an mRNA template for translation. This group includes for example the viruses of the *Picornaviridae* family, with such familiar members as *Human rhinovirus A*, a causative agent for the common cold, and the hopefully soon eradicated *Poliovirus*.

V Single-stranded (-)sense RNA viruses, such as the viruses of the *Orthomyxoviridae*, including the influenza viruses, and *Rhabdoviridae* families.

VI Single-stranded (+)sense RNA viruses with a DNA intermediate in their life-cycle. The most famous member of the *Retroviridae* family is most probably *Human immunodeficiency Virus 1* (HIV-1) of the genus *Lentivirus*. Even though the genome of retroviruses is (+)sense, it is not used directly as mRNA, but as a template for reverse transcription into DNA.

VII Viruses with double-stranded DNA with an RNA intermediate, such as the viruses of the *Hepadnaviridae* and *Caulimoviridae* families. The genome of the *Hepadnaviridae* is mainly dsDNA, but contains gaps of single-stranded regions. First the ssDNA regions are repaired to dsDNA for transcription to occur. The mRNA is packaged into viral core particles, in which it is reverse transcribed into DNA. Concurrent with reverse transcription into DNA, the RNA template is degraded.

In linear genomes, the genome ends may be covalently attached to terminal proteins, or contain hairpins or other secondary structure elements. For example, the linear dsDNA genome of adenovirus contains terminal proteins covalently attached to the 5'ends of the DNA (Desiderio and Kelly, 1981), whereas the poxviruses have terminal hairpin loops that are cross-linked at the ends (Baroudy et al., 1982). The dsDNA genome of herpesvirus is linear when within the viral capsid, but circularised upon infection (Strang and Stow, 2005).

1.2 Virus structure

The genomes of viruses, be they RNA or DNA, are enclosed within a protective protein shell, or capsid. However, the encapsidation of viral genomes into capsids presents a dilemma: mature virions need to be stable and tolerate a broad range of environmental stress, but when a suitable host cell is encountered, the capsids need to be rapidly disassembled or the genome otherwise made available for a new round of infection. To achieve this, viral capsids have evolved into *metastabile* structures, capable of both protecting the genome but also of rapidly releasing it when appropriate. Viral capsids are not just containers for nucleic acid, but in most cases virus capsids perform several functions: in addition to protecting the viral nucleic acid from degradation by chemical, biological or physical factors, the capsids may also contain structures relevant for vector and/or host cell identification and binding, injection devices for nucleic acid transport into the cell, and for example proteins needed for host cell lysis or modulation of the host immune response.

Based on their shape, viral capsids can be roughly divided into three categories: viruses with helical symmetry, i.e. rod-like or filamentous viruses, such as the tobacco mosaic virus (TMV), viruses with icosahedral symmetry (spherical viruses), such as the adenoviruses or picornaviruses, and viruses that display no clear symmetry or are even totally amorphous, for example the poxviruses. Examples of different types of viral capsids and virion structures are shown in Fig. 1.

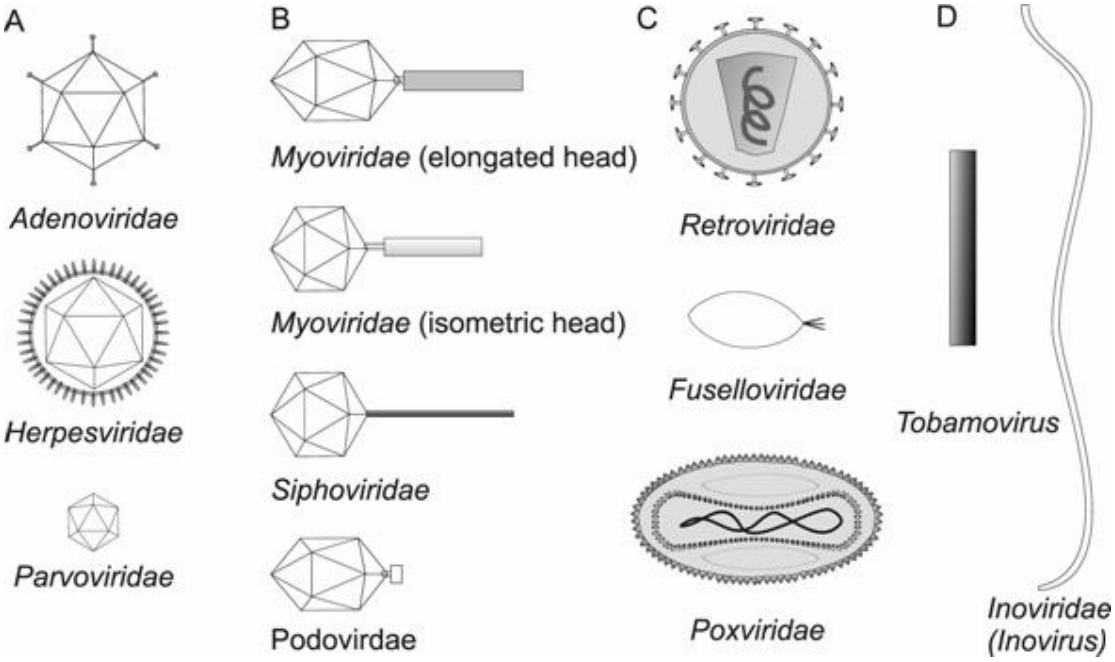

Figure 1. Examples of different types of virion structures
A) Icosahedral symmetry, B) Binary symmetry, C) Only partially symmetric or amorphous viruses, D) Helical symmetry. Based on The VIII ICTV Report on Virus Taxonomy, Fauquet et al., 2005. (Not in scale).

Combinations of the above described classes can also be found. Tailed bacteriophages present so-called binary symmetry: they have icosahedral heads (or elongated icosahedrons), with fiber-like tails attached at one vertex of the icosahedron. The tailed phages (order *Caudovirales*) can further be divided into three families on the basis of the type of their tail: long contractile (*Myoviridae*), long but non-contractile (*Siphoviridae*), and short (*Podoviridae*) tails. The *Geminiviruses* have capsids formed of two joined icosahedral particles. Recently, new types of viruses with curious capsid structures have been found from extremophiles of the domain Archaea, especially the Crenarchaeota, for example the acidianus bottle-shaped virus (ABV) representing the new *Ampullaviridae* family (Häring et al., 2005a), the sulfolobus neozealandicus droplet-shaped virus (SNDV) of the *Guttaviridae* (Arnold et al., 2000) and the lemon-shaped particles of sulfolobus spindle-shaped virus 1 (SSV1) belonging to the *Fuselloviridae* family (Martin et al., 1984).

The paradigm for virus capsids has been that once matured and outside the cell, virus capsids are inert particles until a new host is encountered. The archaeal acidianus two-tailed virus (ATV) challenges this view: particles emerging from cells are lemon-shaped and have no tails, but after leaving the cell within hours or days, depending on the temperature, the lemon-shaped particles develop two tails, one at each end of the particle (Häring et al., 2005b; Prangishvili et al., 2006). It has been suggested that this late development of tails may be a strategy to ensure that in the extremely harsh environments where ATV and its host reside, the infective, and maybe less stable, form is adopted only in conditions where susceptible hosts may be available (Häring et al., 2005b).

Many virus capsids are further enveloped with a lipid bilayer. Such envelops are usually obtained either by budding out of the cell, or by budding into the cytoplasm from cell organelles. Viruses of the *Tectiviridae* carry lipid membranes beneath their protein capsids (Bamford, 2005). As an envelope to most virologists brings to mind a membranous structure *surrounding* the protein capsid, it may be slightly misleading to call the tectiviruses or other such viruses enveloped. To avoid confusion, in this thesis, such viruses will be called internal membrane viruses, distinguishing them from 'regular' enveloped viruses.

Viral capsids are formed of multiple copies of one or more capsid protein. This is an efficient way of using the coding capacity of the genome (Crick and Watson, 1956), as coding for a larger protein, would increase the genome size and again lead to the need to increase the capsid size. For example in the tobacco necrosis satellite virus, one capsid subunit is formed of a polypeptide of approximately 200 aa, and the whole genome is only 1240 nt. If every 60 subunits were to have their own gene, the coat proteins alone would require 36 000 nt! Using multiple copies of the same protein may also help in reducing errors in capsid formation, as controlling the production and assembly of a large number of small identical subunits may be easier than that of one or two very large molecules (Crick and Watson, 1956; Caspar and Klug, 1962).

1.2.1 Viruses with helical symmetry

Some viruses are rod-like or filamentous in their appearance, i.e. they have helical symmetry. The classical example of helical viruses is TMV. (For a relatively recent review on TMV structure and assembly see Klug, 1999).

Helical capsids are built from a regular helical array of identical protein subunits, which surround a single nucleic acid molecule wound as helix, possibly with minor coat proteins at the ends of the structure. Helical structures can be depicted in terms of the number of structural units per turn of helix (μ), and he axial rise per unit (ρ), which give the pitch of the helix (P), ($P=\mu \times \rho$), as described in the early structural studies of TMV (Franklin, 1955; Crick and Watson, 1956). The length of a helical structure is not defined by the coat protein structure, but can be varied according to the length of the genome in case, as suggested already by Crick and Watson (1956). A schematic picture of the structural principle of a helical virus is presented in Fig.2., and assembly of viruses with helical symmetry will be described in section 1.3.2.

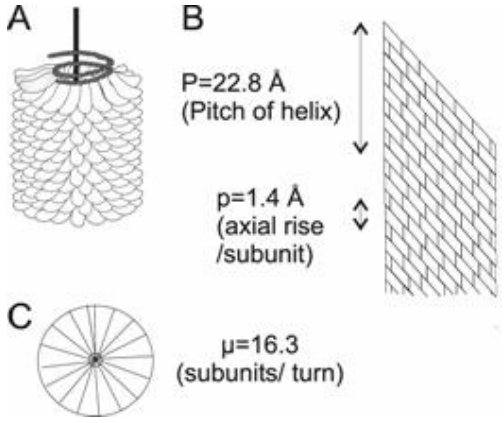

Figure 2. Structure of helical viruses.
A) Protein subunits are wound around nucleic acid to form the virion. B) and C), The virion can be described in terms of the axial rise per unit (p) and the number of structural units per turn of helix (μ), giving the pitch of the helix (P). (Panel A adapted from Klug, 1999, panels B and C from: BS109 Virus Structure, http://www.tulane.edu/dmsander/WWW/109/structure.html.)

1.2.2 Icosahedral viruses

Many viruses enclose their genomes in icosahedral protein shells. An icosahedron is a classical platonic solid formed of 20 triangular facets, joined at 12 vertices and 30 edges, representing 3-fold, 5-fold and 2-fold symmetry, respectively (see Fig. 3a). Due to the inherent asymmetry of proteins, a minimum of three asymmetric protein subunits (or *asymmetric units*) are needed to form one triangular facet, i.e. the minimum number of capsid protein units needed to form an icosahedral structure is 60. However, 60 subunits cannot produce a very large structure. When a larger icosahedral structure is needed, capsid size is not increased by making the capsid proteins larger, but by adding more copies of the same protein. However, when more than 60 subunits are used to build an icosahedral capsid, the subunits can no longer be in identical structural environments (Caspar and Klug, 1962). I.e. in a capsid with 60 subunits, each subunit is part of a pentamer surrounding a vertex, but in larger capsids, all subunits cannot occupy the 5-fold positions, and the capsids are built of pentameric structures at the vertices and hexamers at other positions. The classical quasi-equivalence theory by Caspar and Klug (1962) states that in such icosahedral capsid structures with more than 60 subunits, the non-covalent bonding properties of subunits in different structural environments are similar, but not identical, and thus the subunits occupy *quasi-equivalent* positions in the capsid.

The architectural principles by which icosahedral capsids are built can be described in terms of their triangulation (T) number (Caspar and Klug, 1962). The T-number denotes the number of multiples of 60 of the asymmetric unit needed to

assemble a particular capsid, and thus also describes the amount and arrangement of structural units per facet. The total number of subunits is thus 60T, and a capsid that is formed of only 60 subunits is described to have a T-number of 1 (T=1), or for example, if 180 subunits are used, T=3. As the T-number can be thought of as a subdivision of the triangular facets into smaller triangles, geometry dictates that only certain T-values are allowed. The T-number is described by the following formula: $T=h^2+hk+k^2$, where h and k are integers describing the spacing and spacial relationship of the pentamers (at the capsid vertices), separated by hexamers, in the surface lattice of the viral structure. The geometric principles for producing quasi-equivalent capsid structures are presented in Fig. 3e, and capsid structures with T-numbers 1, 3 and 25 are presented in Figs. 3b, c, and d, respectively.

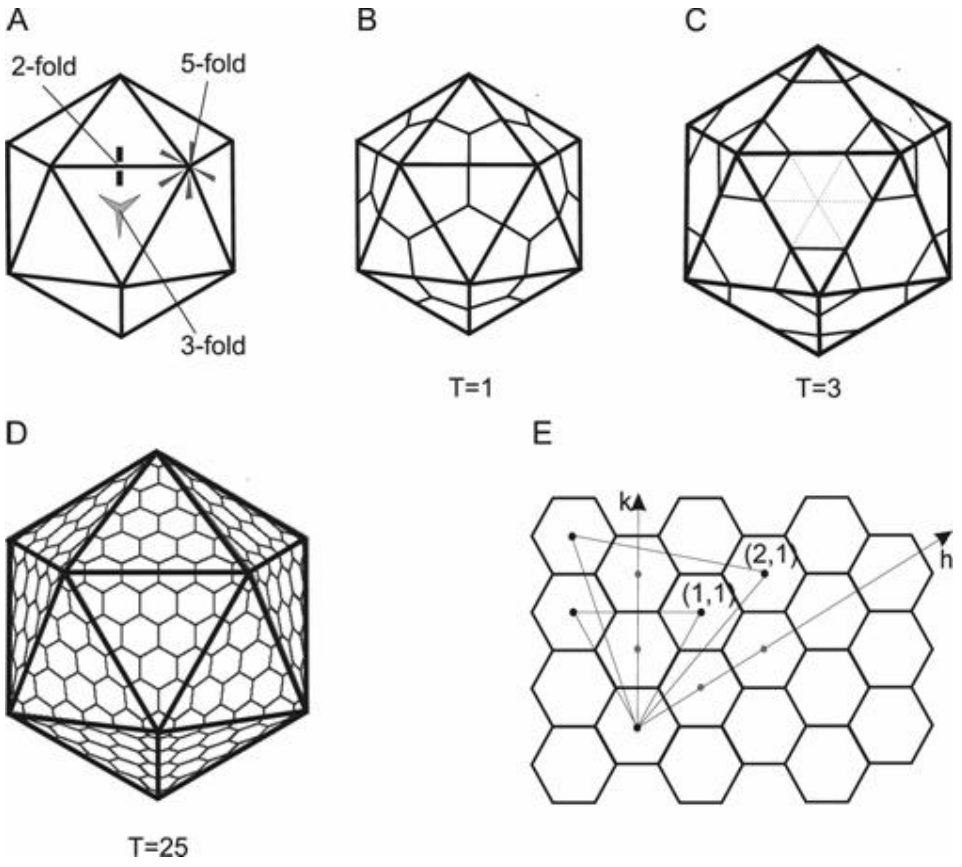

Figure 3. Icosahedral virus structure.
A) An icosahedron, B) a T=1 capsid, C) a T=3 capsid, D) a T=25 capsid. E) The geometric principle for the formation of surface lattices for icosahedral structures. The surface lattice is initially shown as a net of planar hexagons. A closed icosahedral capsid can be generated by replacing 12 of the planar hexamers with convex pentamers at appropriate positions. For a facet of a particular surface organisation and T-number with known h and k values, a model of one facet can be generated by as follows: the origin (h,k = 0,0) and the hexamer described by (h, k) are replaced by convex pentamers, the third pentamer of the triangle identified by three-fold symmetry. As an example, surface lattices for T=3 (h,k=1,1) and T=7 (h,k=2,1) capsids are shown. (As presented in the Virus particle explorer (Viper) database, Shepherd et al., 2006)

Viral capsids studied so far range from simple T=1 capsids, seen for example in the small ssDNA canine parvovirus (Tsao et al., 1991), to very large capsids with T-numbers as high as 169 for chilo iridescent virus (CIV) (Yan et al., 2000) and 219 for the marine algal virus phaeocystis pouchetii virus, PpV01, (Yan et al., 2005), or even more, as suggested for the huge, 5000 Å in diameter mimivirus capsid for which a T=number of 1179 has been estimated (Xiao et al., 2005a).

Instead of using only one type of capsid protein, viruses may use two or more types of structurally similar proteins to build their capsids: often, in viruses with a T-number higher than one, pentameric positions are occupied by a second type of protein, often structurally similar to the main capsid protein, or, the capsomers may be formed of several different protein species. In such a case, the T-number is often referred to as a 'pseudo T-number'. The former is a very common principle in viruses, e.g. in the T=25 adenovirus capsid the 5-fold positions are occupied by pentameric penton proteins, and the other positions by hexameric hexons (Burnett, 1985; Stewart et al., 1991; Stewart et al., 1993). Interestingly though, the hexon proteins of adenovirus are not actually hexamers of single protein subunits, but trimers, each hexon monomer having a 'dimeric' double-β-barrel structure, the trimer thus being a structural hexamer of six β-barrels (Roberts et al., 1986; Stewart et al., 1993). In picornaviruses (e.g. poliovirus and rhinoviruses), the asymmetric unit of the capsid is a heterotrimer of proteins VP1, VP2 and VP3 (Hogle et al., 1985; Rossmann et al., 1985).

In tailed bacteriophages, the head portions display either regular icosahedral symmetry, or the heads may be elongated, prolate, with otherwise icosahedral symmetry, but having one or more additional layers of hexamers inserted into the 'equator' of the capsid, as in bacteriophage T4 (Fokine et al., 2004).

There are other exceptions to the classical Caspar and Klug quasi-equivalence theory: for example, even though two is a 'forbidden' T-number, LA-virus (Naitow et al., 2002), members of the *Reoviridae* (Grimes et al., 1998; Hill et al., 1999; Nakagawa et al., 2003) and the cystovirus phi6 (Huiskonen et al., 2006) have peculiar inner capsid shells, core particles, with 120 proteins, where asymmetric dimers of the capsid protein make up one structural unit.

The capsid may also be stabilised by cementing or glue proteins, i.e. minor capsid proteins that stabilise the capsid structure and plug holes between the main capsomers. For example, the cementing protein IX of adenovirus provides stabilising contacts between the major capsid protein hexons (Furcinitti et al., 1989; Stewart et al., 1991).

Naturally, virus capsids carry many other proteins: these may be proteins involved in DNA packaging or ejection, receptor binding, or other parts of the infection process. The incorporation of these proteins is strictly regulated to assure their correct amount and position in the virion, and they must be included in the structure at the correct assembly stage. Virus capsids may also contain so-called decoration proteins. These are proteins on the capsid structure which are not necessarily essential for capsid assembly, stability or infection. Presumably, these proteins have more than just a 'decorative' function, at least in certain circumstances, but these functions have just not been identified yet.

1.2.3 Glycosylation and other modifications in viral capsid proteins

Virion proteins of animal and plant viruses may be modified in many ways: phosphorylated, glycosylated, methylated, myristoylated, palmitoylated, or sulfated (Goddard et al., 1989; Edson, 1993; Burton KS, 1996; Fang et al., 1999; Chen et al., 2005; Liao et al., 2006; Seddas and Boissinot, 2006), or they may undergo specific proteolytic processing (Ait-Goughoulte et al., 2006). In bacteriophages, other post-translational modifications than proteolytic processing of capsid proteins are rare. For example, in the case of bacteriophage HK97, proteolytic processing in conjunction with covalent cross-linking of the capsid proteins is essential for virion maturation (Duda et al., 1995a). In most cases, viruses use their host organism's post-translational modification machinery, but in some cases, viruses may also encode their own modifying enzymes: paramecium bursaria chlorella virus 1 (PBCV-1) was the first virus to be found to have its own glycosyltransferase enzyme which is used to glycosylate its major capsid protein (Graves et al., 2001).

1.2.4 Mature capsids are not static structures

Although mature virions are very stable structures, resistant to many environmental stress factors, this does not mean that they are completely static. Naturally, receptor binding structures of viruses are often very flexible, but in addition, so may the main capsid structure be too. Actually, surprisingly large changes and fluctuations ('breathing') may occur in virus capsids without compromising protection of the nucleic acid inside. Such dynamic behaviour may even be a prerequisite for the infectivity of certain viruses. For example, the N-terminal region of the poliovirus VP1 capsid protein, which in the X-ray structure is buried, was found to be able to bind to antibodies, leading to the suggestion that this region could alternate between two different positions, inside and outside the capsid (Roivainen et al., 1991; Roivainen et al., 1993). The N-terminus of VP1 was later shown to be able to attach to liposomes, and was suggested to insert into endosomal membranes and thus to play an important role in poliovirus entry (Fricks and Hogle, 1990)

Similar fluctuations of VP1 and VP4 capsid proteins have been observed in another picornavirus, human rhinovirus 14 (HRV14): exposure of particles to proteases and subsequent analysis with matrix-assisted laser desorption/ ionisation mass-spectrometry (MALDI-MS) showed that regions internal in the X-ray structure were highly susceptible to protease cleavage in solution (Lewis et al., 1998). Further treatment of the HRV14 capsids with a drug stabilising the capsid led to inhibition of the exposure of the normally fluctuating VP1 and VP4 termini and inhibition of infection by preventing uncoating of the viral RNA (Lewis et al., 1998).

1.3 Capsid assembly

There are two basic types of mechanisms for capsid assembly and genome encapsidation: First, the genome and capsid proteins can co-condense to form virus particles ('coassembly'). The second option is to first form empty (icosahedral) particles, called procapsids (also called proheads or preheads) into which the viral genome is then specifically inserted. The latter mechanism is used by dsDNA viruses such as the tailed bacteriophages and herpesviruses, dsRNA viruses, e.g. the cystoviruses, and ssDNA viruses such as the microviruses.

The assembly of virus capsids is an inherently 'self-directed' event: the information needed for building a capsid is already present in the amino acid sequence and three-dimensional structure of the capsid proteins (Caspar and Klug, 1962). The energy required for assembly is typically intrinsic to the process, 'stored' in the conformation of the interacting subunits (Caspar and Klug, 1962; Steven, 1993). The assembled structure is at an energy minimum compared to the free subunits, and the process of assembly is essentially driven by the laws of thermodynamics (Caspar and Klug, 1962).

Often though, accessory proteins are involved in capsid assembly. In the case of preformed procapsids there are often scaffolding proteins that are a structural part of the procapsid aiding in the assembly of the protein shell, but are discarded during subsequent virus maturation. Non-structural assembly factors that are necessary for the assembly stage but are never an integral part of the procapsid or mature virion may also be involved. These may be proteins of either viral or cellular origin.

1.3.1 Assembly of preformed procapsids

Even though the three-dimensional structures of the capsid proteins contain the information needed for assembly, and the finished structure itself is in a lower energy state (Steven, 1993), reaching this state may not be thermodynamically favourable due to high-energy intermediates. Therefore, the presence of capsid proteins alone may not lead to efficient and high-fidelity assembly of correctly shaped and sized capsids. The assembly of icosahedral capsids thus often involves specific virus-encoded scaffolding proteins. Scaffolding proteins may be either internal or external. External scaffolding structures are usually icosahedrally ordered, and form cage-like structures around the forming capsids. Internal scaffolding proteins may form core-like structures within the procapsid that are icosahedrally ordered, as in the case of the D protein of the small bacteriophage phiX174 (Ilag et al., 1995; Dokland et al., 1997). Alternatively, the scaffolding structures may present only local icosahedral symmetry at certain areas of interaction between the scaffolding and capsid proteins, as in the case of the larger P22 bacteriophage (Thuman-Commike et al., 1996). In bacteriophage P22, for example, the amount of scaffolding protein may vary, and strict stoichiometry is not required for assembly of capsids, as long as the amount of scaffolding protein is above a certain threshold level (Prevelige et al., 1988). Although in the procapsid structure, internal scaffolds may be reminescent of a core, preformed core particles are not formed in dsDNA bacteriophage assembly, and in most cases, scaffolding proteins bind to the coat proteins as mono-, di- or tetramers (Prevelige et al., 1988; Prevelige et al., 1993; Lee and Guo, 1995b; Cerritelli and Studier, 1996). The exception to this is the large, prolate bacteriophage T4, which has been shown to assemble a prolate core particle consisting of scaffolding protein gp22, in addition to several other proteins, around which the capsid can then be built (Black and Brown, 1976; Traub et al., 1981; Traub et al., 1984; Traub and Maeder, 1984; Kuhn et al., 1987).

Scaffolding proteins may function in several ways (for reviews on the subject, see Dokland, 1999; Fane and Prevelige, 2003): scaffolding proteins may be needed to overcome the 'nucleation barrier' of the capsid proteins due to the fact that the statistical probability of getting sufficient amounts of capsid protein subunits together in space and time to initiate capsid assembly may be very low. The scaffolding proteins may also help by promoting a conformational change in the capsid protein subunits from assembly-incompetent to assembly-competent form in order to allow assembly. In addition to lowering the nucleation barrier, scaffolding proteins may be needed for efficient completion of the reaction. Scaffolding proteins may not only ensure correct assembly of the coat proteins, but also present a mechanism for the controlled timing of assembly, as in most cases, procapsid formation is extremely inefficient in the absence of scaffolding.

Capsid proteins are often able to assemble in several different ways, with similar local bonding interactions but subtle alterations in the relative positions of the subunits. This can produce a variety of alternative morphological forms. Scaffolding proteins may be in place to ensure that only the 'correct' form is assembled. For example in the case of the T=7 bacteriophage P22, scaffolding affects both the efficiency and accuracy of assembly. In the absence of scaffolding proteins, only very few capsids are assembled within the normal assembly time-scale, but if assembly without scaffolding proteins is allowed to proceed longer, both T=4 and T=7 capsids, in addition to spiral structures, are formed (Earnshaw and King, 1978; Thuman-Commike et al., 1998).

In the case of bacteriophage P2 and its satellite phage P4, regulation of the formation of different capsid forms has been taken even further: The bacteriophage P2 capsid protein gpN alone is capable of forming capsids of two sizes 45 nm (T=4) and 60 nm (T=7) in diameter, albeit at low efficiency (Marvik et al., 1994). The P2 scaffolding protein gpO promotes the efficient assembly of the larger T=7 capsid (Marvik et al., 1994; Wang et al., 2006). Satellite phage P4 expresses gpSid, an external scaffolding protein forcing the P2 gpN capsid protein to assemble into smaller T=4 particles which are able to accommodate only the smaller P4 genome but not the longer P2 genome (Dokland et al., 1992; Marvik et al., 1994; Marvik et al., 1995).

Scaffolding proteins may also be important to ensure the correct incorporation of the portal ring needed for DNA packaging and other minor capsid components. In bacteriophage P22, scaffolding protein has been shown to interact with both the major coat protein and the portal protein, and in the presence of mutant scaffolding protein, both portal and minor DNA injection proteins fail to be incorporated (Bazinet and King, 1988; Weigele et al., 2005). Also in herpes simplex virus 1 (HSV-1), scaffolding protein has been shown to bind portal protein and to be involved in the initiation of assembly (Newcomb et al., 2003; Newcomb et al., 2005). Interestingly, in both cases, although the portal seems to be involved in the initiation of capsid assembly, and cannot be incorporated at a later stage, assembly of both P22 and HSV-1 capsid-like particles can occur in the absence of portal, although these structures cannot mature to normal DNA-containing virions (Bazinet and King, 1988; Newcomb et al., 2005; Weigele et al., 2005). However, although bacteriophage P22 coat protein and scaffold alone are able to produce proper T=7 structures *in vitro* (Prevelige et al., 1988), the P22 portal does seem to be involved in regulating capsid assembly *in vivo*, or, at least the correct amount of portal protein is extremely important. Over-expression of the P22 portal protein was shown to cause formation of aberrant procapsid-like particles that had T=7 symmetry, but contained twice the normal amount of portal protein and were unable to package DNA (Moore and Prevelige, 2002). Additionally, aberrant spiral structures, as well as smaller than normal T=4 particles, which surprisingly were able to package a headful of DNA, were also formed (Moore and Prevelige, 2002). In both bacteriophages SPP1 and phi29, portal protein in similarly important for the formation of correctly sized particles and in the absence of portal, particles of aberrant sizes and shapes are produced,

even though scaffolding protein is present (Guo et al., 1991; Dröge et al., 2000).

Even though in most cases, the portal protein has been suggested to act with scaffolding to initiate assembly, quite the opposite has been suggested for bacteriophage T7, where capsid assembly has been suggested to be terminated by closure of the incomplete proheads by the connector and core complexes (Cerritelli and Studier, 1996).

The classical example of using both internal and external scaffolds is presented by the T=1 microvirus phiX174 (for a recent review, see Fane and Prevelige, 2003): The procapsid of phiX174 contains 60 copies each of coat protein F and spike protein G, 60 copies of internal scaffolding protein B, and 240 copies of external scaffolding protein D (Dokland et al., 1999). The first assembly intermediates seen in phiX174 assembly are homopentamers of coat protein F and spike protein G, (the 9S and 6S complexes, respectively) (Tonegawa and Hayashi, 1970). Assembly of phiX174 then proceeds by binding of five copies of the internal scaffolding protein B to the 'inner' side of a coat protein pentamer, causing a conformational change converting the capsid protein to an assembly-competent form (Dokland et al., 1997; Dokland et al., 1999; Novak and Fane, 2004). The conformational change in the capsid protein and the presence of scaffolding protein B now allow the complex to bind the major spike protein (G) and the external scaffolding protein D.

External scaffolding protein D mediates contacts between coat protein pentamers and between coat protein and spikes, and allows the formation of icosahedral procapsids (Fujisawa and Hayashi, 1977b; Dokland et al., 1997; Dokland et al., 1999)

The N-terminus of the phiX174 internal scaffolding protein has been suggested to induce the conformational change in the coat protein and facilitating spike incorporation (Novak and Fane, 2004). The C-terminus of the scaffolding protein, and especially a set of aromatic amino acid residues in it, are the most important region for mediating the interaction with the capsid protein, and determine the specificity of the scaffolding protein, as studied by cross-complementation of chimeric scaffolding proteins (Burch and Fane, 2000). The significance of the scaffolding protein C-termini in determining specificity may be a common phenomenon in capsid assembly, as for example the C-terminal parts of the scaffolding proteins of herpesviruses and bacteriophage P22 have similarly been recognised as important for capsid protein interaction and specificity determination (Oien et al., 1997; Preston et al., 1997; Parker and Prevelige, 1998).

After formation of the procapsid, the internal scaffolding proteins need to be removed from the capsid interior in order to accommodate the genome. In many tailed phages the scaffolding protein is cleaved by a viral protease to allow exit, whereas for example in bacteriophage P22 the scaffolding proteins are removed as intact proteins and can be recycled for further assembly of particles (Prasad et al., 1993). The latter mechanism probably requires the scaffolding protein to be partially unfolded in order for it to be extruded through the relatively small openings between the capsid proteins available for exit. In phiX174, removal of the internal scaffolding protein B has been suggested to occur as a consequence of competition for binding sites on capsid protein F between the scaffolding protein and the DNA complexed with DNA binding protein J (Dokland et al., 1999). Electrostatic interactions have similarly been suggested to be involved in bacteriophage P22 scaffolding protein removal and capsid maturation (Parker and Prevelige, 1998).

In bacteriophage HK97 there is no separate scaffolding protein, and the capsid protein alone is able to efficiently assemble correctly sized and shaped particles (Duda et al., 1995b). Instead, in HK97 a 102 aa domain of the capsid protein (the delta domain), behaves similarly to a scaffolding protein and mediates assembly, and is subsequently cleaved and removed from the particle (Duda et al., 1995a).

In most icosahedral viruses, external scaffolding proteins are not seen. However, many viruses have stabilising proteins in their capsids that function similarly to external scaffolds but are not removed from the final virion structure. In the HSV-1 procapids, there is little or no contact between the coat protein subunits of adjacent capsomers (Trus et al., 1996). During assembly of the T=16 herpesvirus capsid, triplex proteins consisting of a heterotrimer of 2 molecules of VP23 and one molecule of VP19, bind to major capsid protein-scaffolding protein complexes (Spencer et al., 1998). The triplex proteins bind between the capsomers to connect them and allow procapsid assembly (Trus et al., 1996). In the mature virion, more direct contacts between the capsomers are formed, although the triplex proteins still stay bound to the capsid and stabilise it (Newcomb et al., 1996; Trus et al., 1996). In the absence of VP19 no capsids are formed, whereas the absence of VP23 leads to formation of aberrant particles (Thomsen et al., 1994; Rixon et al., 1996).

In the tailed phages, formation of the icosahedral head is not the only complex assembly reaction in the phage life cycle. Assembly has been found to be divided into several ‘assembly lines’: in addition to head assembly, the assembly of the tail fibers and the tail form their own assembly lines, the products of which are subsequently combined with the assembled head to give the mature virus.

1.3.2 Formation of viral particles via coassembly

Formation of viral particles by a coassembly reaction is governed by nucleic acid - protein interactions, in addition to interactions between the coat protein subunits. Coassembly reactions are used to form both helical and some icosahedral viruses.

As described earlier, helical capsids are built from a regular helical array of identical protein subunits surrounding a single nucleic acid molecule. Although the coat protein subunits of a growing helical structure could in theory be added to the nucleic acid as monomers or dimers, often, more complex multimers are seen. In TMV, coat protein subunits assemble into two-layered disc-like structures of 34 protein subunits (see Fig. 4), which further assemble into rod-like structures around the (+)strand RNA genome (reviewed in Klug, 1999). Despite its apparent simplicity, the assembly of TMV is very specific: assembly is initiated by binding of the two-layer disk of coat proteins to a specific initiation tract, called the origin of assembly, approximately 1 kb from the 3' end of the RNA genome (Zimmern and Wilson, 1976; Zimmern and Butler, 1977), preventing host RNA's not containing this tract being incorporated. The disc dislocates (see Fig 4.) to expose subunits that can bind to the next disc assembled to the growing structure. From this point, assembly proceeds in both 5' and 3' directions. The length of the genome is not always the only defining factor in the length of a helical capsid. In filamentous bacteriophage fd, the minor coat protein pIII, is necessary for termination of assembly, and in its absence, longer than normal particles are produced, and the particles cannot be released from the cell membrane, their site of assembly (Rakonjac and Model, 1998).

Initiation of coassembly of icosahedral structures is similarly mediated by interactions between the nucleic acid and capsid proteins. For example, in the T=3 bacteriophage MS2, a dimer of the coat protein has been found to specifically bind to a stem-loop structure in the viral ssRNA genome, repressing translation and probably acting as an assembly trigger (Carey et al., 1983; Romaniuk et al., 1987; Peabody, 1993). This stem-loop structure is the only signal needed for RNA encapsidation and heterologous RNAs with the MS2 stem-loop structure can be encapsidated to form virus-like particles (VLPs) (Legendre and Fastrez, 2005).

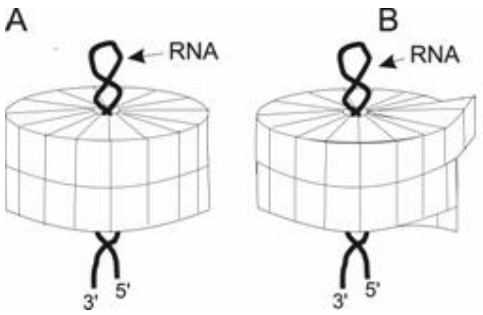

Figure 4. Assembly of TMV and similar helical viruses.
A) Assembly is initiated by binding of a two-layer disk of coat proteins to a specific initiation tract approx. 1 kb from the 3' end of the RNA genome. B) The disc dislocates to give free subunits that can bind to the next disc assembled to the growing structure and assembly can proceed in both 5' and 3' directions. (Adapted from http://www.vu-wien.ac.at/i123/ALLVIR/SELFASSEMB1.HTML, University of Vienna.)

1.4 Packaging genomes into preformed particles

Encapsidation of viral genomes within a preformed icosahedral protein capsid presents a unique set of challenges: Firstly, the genomes of icosahedral viruses are enormous in length compared to the inner diameter and volume of the capsids they are to occupy. Packaging DNA or RNA to such a high density is energetically extremely unfavourable. Secondly, the nucleic acid needs to be topologically organised so that when a suitable host cell is encountered the genome can efficiently be transferred into the cell and a new round of infection initiated. Specificity is an additional challenge, as energy and material should not be wasted for non-productive packaging of host DNA or RNA into the viral capsids.

The mechanism of using preformed particles is best characterised in the tailed bacteriophages. The same mechanism has also been found to be used by the herpesviruses, but may be used by other eukaryotic viruses and non-tailed dsDNA icosahedral bacteriophages as well. DsRNA viruses and certain ssDNA viruses have also been shown to use preformed particles, although their packaging mechanisms differ significantly from that of the tailed phages and herpesviruses.

1.4.1 Genome packaging in dsDNA viruses: tailed phages and herpesviruses

Packaging of dsDNA genomes into preformed icosahedral capsids occurs through one specific vertex of the icosahedral capsid. This mechanism was first characterised for tailed dsDNA bacteriophages, but a unique packaging vertex has been described for HSV-1 as well (Newcomb et al., 2001). The packaging vertex contains a ring-like structure called the portal, through which the DNA is threaded into the particle. In tailed bacteriophages the portal also acts as the tail attachment site and as the channel through which DNA is translocated into the host cell. Chemical energy stored in ATP molecules is converted to mechanical energy and the DNA is translocated into the capsid by an enzyme called a packaging ATPase or terminase, performing also DNA cleavage in the case of viruses with concatemeric DNA. A generalised scheme for DNA packaging in tailed bacteriophages is presented in Fig.5.

Packaging is not an isolated process in the phage life cycle. In at least bacteriophage T4, DNA packaging has been shown to be coupled to replication and transcription: DNA cutting by the terminase occurs preferentially on transcriptionally active DNA (Bhattacharyya and Rao, 1993) and late transcription-replication machinery proteins have been shown to stimulate packaging (Black and Peng, 2006). In bacteriophage T7, packaging has been suggested to be initiated by interaction of the transcription elongation complex, paused at a specific consensus site at the right end of the DNA concatemer junction, with the prohead-terminase complex (Zhang and Studier, 2004).

Due to the location of the packaging machineries of dsDNA phages at a single vertex of the otherwise icosahedrally symmetric capsid, using conventional structure determination methods involving icosahedral averaging to study DNA packaging is rather pointless. Recently, several asymmetric single-particle cryo-EM structures of mature dsDNA bacteriophages and procapsids have been published, providing important information on the organisation of packaging machineries within their proper context in the capsid (Agirrezabala et al., 2005a; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006).

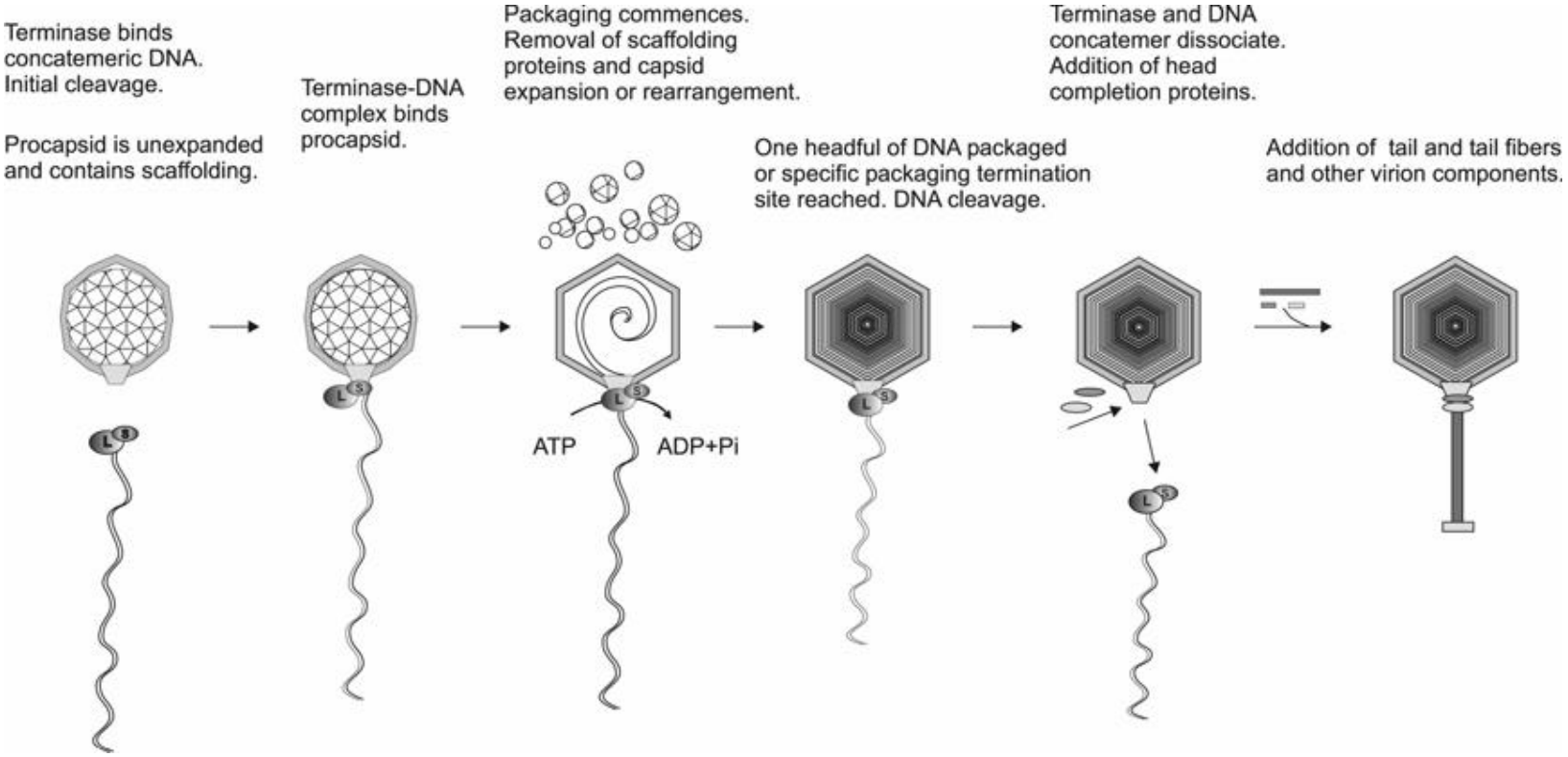

Figure 5. A generic pathway for dsDNA packaging in tailed phages.

In vitro packaging systems have been especially useful for studying bacteriophage packaging. *In vitro* systems have been developed for various different tailed bacteriophages representing different packaging mechanisms: A) phages using terminases that cut and package the DNA in a headful manner, such as P22 (Poteete et al., 1979; Gope and Serwer, 1983), SPP1 (Dröge and Tavares, 2000; Oliveira et al., 2005), T4 (Black, 1981; Leffers and Rao, 2000; Malys et al., 2002; Black and Peng, 2006), and T1 (Liebeschuetz et al., 1985), B) phages cutting their concatemeric DNA only at specific cut sites, such as lambda (Hwang and Feiss, 1995; Yang and Catalano, 2003; Gaussier et al., 2006), T3 (Hamada et al., 1986), and T7 (Kerr and Sadowski, 1974; Masker and Serwer, 1982), and C) phages having unit-length DNA genomes that are not cleaved during packaging, such as phi29 (Bjornsti et al., 1981; Guo et al., 1986; Grimes and Anderson, 1989). These systems either use extracts from cells infected with mutant viruses producing empty procapsids, or as in the more sophisticated ones, all components are purified, providing a more accurate picture of the components involved and the mechanisms in place. In some cases, the systems even involve *in vitro* assembly of procapsids from recombinant proteins, as for example in the case of bacteriophage phi29 *in vitro* packaging and assembly (Lee and Guo, 1994). However, in most *in vitro* systems, purified procapsids or extracts of mutant procapsids and packaging proteins are used. It should be noted that most of the *in vitro* packaging assays, especially those for tailed phages, where assembly of tail components may be inefficient *in vitro*, are actually 'nuclease protection assays' that measure only encapsidation of the DNA and protection from nucleases. Such assays do not show whether the DNA is productively packaged and later capable of ejection and initiation of infection. No *in vitro* packaging system has yet been described for a membrane-containing DNA virus.

1.4.1.1 Portals

Portals, or connectors as they are also known, provide the entry site for DNA into the capsid, and also an attachment site for the terminase (Yeo and Feiss, 1995; Lin et al., 1999) and other DNA packaging proteins (Stiege et al., 2003) and later, for head completion proteins and tail components (Lurz et al., 2001; Orlova et al., 2003; Isidro et al., 2004; Fokine et al., 2005a). Portals are cone or doughnut-shaped homo-oligomers of 12 subunits of portal protein, with a central channel large enough to allow the passage of dsDNA, as determined for a variety of tailed bacteriophages (Carrascosa et al., 1985; Carazo et al., 1986; Bazinet et al., 1988; Donate et al., 1988; Valpuesta et al., 1992; Valpuesta et al., 1999; Agirrezabala et al., 2005b). The most detailed portal structure available is that of the bacteriophage phi29 portal (Simpson et al., 2000; Simpson et al., 2001; Guasch et al., 2002). Multimers of 11-15 subunits have been reported for some recombinant portal proteins (Dube et al., 1993; Orlova et al., 1999; Newcomb et al., 2001; Cingolani et al., 2002; Trus et al., 2004), as well as for the HSV-1 virion, as analysed by immunogold-labelling (Newcomb et al., 2001). However, all portals isolated from procapsids or mature virions, as well as portals within the capsid structure, which have been studied by cryo-EM or X-ray crystallography, have been found to be dodecamers of portal protein (Lurz et al., 2001; Agirrezabala et al., 2005a; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006). This may imply that portals with other than 12-fold symmetry are either intermediate states that will upon assembly to capsids lose one subunit, or the products of incorrect assembly and will not be incorporated into the virion, as suggested in (Lurz et al., 2001).

The presence of a dodecameric portal structure at a 5-fold vertex of the capsid creates a symmetry mismatch. This symmetry mismatch has been proposed to allow for rotation of the portal relative to the capsid, thus possibly aiding in DNA packaging (Hendrix, 1978). Although the idea of rotation fits many theoretical models of packaging and does not disagree with what is known of packaging so far, direct experimental evidence for or against rotation has unfortunately been scarce. However, in bacteriophage T4, N- and C-terminal fusion proteins of the portal protein that would effectively block any possible rotation of the portal structure in relation to the capsid were not found to inhibit DNA packaging, suggesting that rotation of the portal is not strictly necessary for packaging (Baumann et al., 2006).

Portals are not just passive conduits for DNA entry and docking sites for terminases, but they may also play a more active role in the packaging process, for example by stimulating terminase activity. In bacteriophage SPP1, the portal protein has been found to modulate the ATPase activity but not the endonuclease activity of the isolated terminase large subunit (Camacho et al., 2003). Further, portal protein embedded in the procapsid was found to stimulate the ATPase activity of the large subunit more than 10-fold, whereas isolated portal protein had a much lesser effect (Oliveira et al., 2006). Mutations in the SPP1 portal protein were similarly found to modulate the ATPase activity of the terminase (Oliveira et al., 2006). In T4, portal protein has also been shown to mildly stimulate the ATPase activity of the terminase large subunit (Baumann and Black, 2003).

Portals have been implicated in both the maturation expansion of particles (Cerritelli et al., 2003), and in triggering the cleavage of DNA once a headful has been packaged (Casjens et al., 1992; Tavares et al., 1992; Orlova et al., 1999; Lander et al., 2006). The latter is supported by the finding that mutations in the portal protein affect the amount of DNA packaged (Casjens et al., 1992; Tavares et al., 1992). Recently, a model for how this triggering of cleavage could be achieved in bacteriophage P22 was presented (Lander et al., 2006): In the asymmetric cryo-EM reconstruction of bacteriophage P22, the DNA in packaged virions was found to be tightly spooled around the upper part of the portal located within the capsid, forcing a conformational change in the portal as compared to the isolated portal structure. The increasing pressure on the portal and the subsequent conformational change caused by the increasingly tight DNA spooling was suggested to be the switch activating the cleavage signal (Lander et al., 2006). Isolated bacteriophage phi29 portals have also been shown to be able to wrap supercoiled DNA around their outside surface (Turnquist et al., 1992). This has been suggested to either be a mechanism to target the phi29 DNA to the procapsid or to possibly act to convert the portal from a static structure to a more dynamic packaging-motor organelle (Turnquist et al., 1992). As discussed in section 1.3.1, portals have also been shown to interact with scaffolding proteins and possibly play an important role in capsid assembly, in both initiation of assembly and form determination

1.4.1.2 Terminases and packaging ATPases

Terminases are heteromers of the small and large terminase subunits. Although terminases have usually been assumed to function as heterodimers, for phage lambda terminase, a heterotrimer of one large subunit and two small subunits has been reported (Maluf et al., 2005). The large terminase subunit has endonuclease and ATPase activities and performs the DNA cleavage, ATP hydrolysis and most likely the actual DNA translocation events needed for DNA packaging, as shown for example for the bacteriophage T4 terminase large subunit (Duffy and Feiss, 2002; Baumann and Black, 2003). Sequence analysis has revealed classical Walker A and Walker B ATPase motifs, typical of the so-called P-loop ATP and GTP binding proteins, in the large subunits of most terminase large subunits of tailed phages and herpesviruses (Walker et al., 1982; Gorbalenya and Koonin, 1989; Saraste et al., 1990; Mitchell et al., 2002). It has also been suggested that T4 gp17 and other terminase large subunits have some features typical of the DEXD/H box helicases family (Linder et al., 1989; Mitchell et al., 2002). An endonuclease domain belonging to the resolvase/integrase/ribonuclease H super-family has been suggested to be located in the C-terminal part of the bacteriophage T5 terminase large subunit (Ponchon et al., 2006). In bacteriophage T4 large terminase subunit gp17, two separate functional domains have indeed been experimentally shown to exist by mutational studies and expression of the N- and C-terminal halves separately as recombinant proteins: the ATPase activity has been localised to the N-terminal half of the protein and the nuclease activity to the C-terminal half (Lin et al., 1999; Rao and Mitchell, 2001; Rentas and Rao, 2003; Kanamaru et al., 2004). The C-terminal and middle portions of the T4 large terminase subunit may be the regions involved in terminase binding to the portal (Lin et al., 1999). Other proteins, such as the T4 portal protein gp20 and ssDNA binding protein gp32 (in the presence of DNA) have also been reported to stimulate the ATPase activity of the large subunit (Baumann and Black, 2003).

The small subunit is responsible for recognition and binding to the specific packaging sites on the phage DNA and probably acts to position the large subunit correctly for cleavage events, as experimentally shown for the lambda, SPP1 and T4 terminase small subunits (Frackman et al., 1985; Shinder and Gold, 1988; Chai et al., 1995; Lin et al., 1997). In bacteriophage lambda, the N-terminal part of the terminase small subunit, gpNu1, has been found to be involved in DNA binding and the C-terminal part interacts with the N-terminus of the large subunit, gpA (Frackman et al., 1985).

The small subunit stimulates the ATPase activity of the large subunit (Gual et al., 2000; Leffers and Rao, 2000; Baumann and Black, 2003). This stimulation is possibly achieved by affecting the multimerisation of the large subunit, as seen in bacteriophage T4 (Leffers and Rao, 2000; Baumann and Black, 2003). Although not strictly required for endonuclease activity, the small subunit of bacteriophage lambda terminase has been observed to stimulate nicking of *cos* sites by the large subunit (Parris et al., 1994; Rubinchik et al., 1994).

Packaging starts with the binding of the terminase enzyme to the concatemeric DNA molecule. Binding and initial cleavage occur either specifically, or semi-specifically. In bacteriophage lambda a highly specific system is in use: terminase first binds to the *cos* site via interaction of the small subunit gpNu1 with three sequence domains (R1-R3) in the *cosB* region of the DNA. This is followed by nicking of the DNA in the central *cosN* region by the large subunit gpA (Feiss and Catalano, 2005). Bacteriophage P22 has a specific *pac* site to which the terminase binds, but the cleavage occurs semi-specifically within a target area approximately 120 bp from this binding site. Bacteriophage Mu does not have concatemeric DNA, but it integrates into the host genome, and has to be excised from the host chromosome for packaging. This leaves non-specific host DNA sequences attached at both ends of the phage genome. The bacteriophage Mu

terminase binds to a specific *pac* site, but the cleavage is performed in a sequence-independent manner within the flanking host DNA. In bacteriophages T3 and T7, the *pac* site contains a promoter for phage RNA polymerase and DNA packaging is transcription-dependent (Hashimoto and Fujisawa, 1992). Packaging of bacteriophage T4 DNA presents additional challenges as the replication of T4 DNA produces a complex branched molecule, which needs to be untangled with assistance from the T4 gp49 resolvase enzyme (endonuclease), for it to be packaged (Golz and Kemper, 1999). No specific *pac* site has been identified in bacteriophage T4, instead, the terminase has been shown to bind preferentially to ssDNA regions in the replication intermediates (Franklin et al., 1998), most probably via components of the replication-coupled late transcription system (Black and Peng, 2006).

After the initial cleavage creating a packaging-competent DNA end, the terminase-DNA complex goes on to bind the procapsid. Packaging is fuelled by ATP hydrolysis performed by the large terminase subunit. In the case of concatemeric DNA, the DNA needs to be cleaved when one genome length has been packaged. This second cleavage can occur either at a specific sequence-determined site such as the lambda *cos* sites, or bacteriophage T3 and T7 *pac* sites, or sequence-independently when a certain amount of DNA is packaged (headful packaging), as occurs in bacteriophages P22, T4 and SPP1. In the headful packaging mechanism, the exact cut-site slightly varies between individual mature virions and packaged genomes may be circularly permuted. For example, in bacteriophage P22, approximately 104% of the genome is packaged per virion, producing terminally redundant ends, which are necessary for DNA replication upon a new infection (Casjens and Hayden, 1988). In bacteriophage Mu the host-derived sequences play a similar role, ensuring that the whole genome can be replicated without losing information from the ends. An interesting example of headful packaging can be seen in bacteriophage T4 mutants. In T4 mutants that produce smaller isometric (so-called 'petite' heads) heads only approximately 40 % of the normal amount of DNA is packaged, but the final DNA density in the filled particle is similar in both mutant and larger wt particles (Eiserling et al., 1970). Correspondingly, mutant capsids larger than wt capsids are able to package significantly larger than normal amounts (Mb range) of DNA (Cummings et al., 1977). Headful packaging is most probably controlled by the portal protein, as described in section 1.4.1.1. The division into *cos* (sequence-specific cleavage) and *pac* (headful cleavage) type enzymes is also reflected in the nucleotide sequences of the corresponding genes (Le Marrec et al., 1997; Lucchini et al., 1999). Unspecific binding of the terminase to host sequences and incorporation of host DNA into the particles, may lead to transfer of cellular DNA by viral particles to another host cell, a process called generalised transduction.

The second (termination) cleavage creates a new packaging-competent end. Terminases and packaging ATPases are usually not a component of mature virions, but associate only transiently with the procapsids to perform the packaging reaction and are then recycled to the next procapsid. After the second cleavage, the terminase-DNA complex dissociates from the capsid and binds to the next procapsid.

In viruses with unit-length genomes no cleavage is necessary and a simple packaging ATPase is sufficient, the best studied example being the packaging ATPase gp16 of bacteriophage phi29 (Guo et al., 1987a). The phi29 genome has covalently attached terminal proteins and replication proceeds by a protein primed mechanism, similar to adenovirus and bacteriophage PRD1 (Salas, 1991). The phi29 terminal protein gp3 is essential for packaging and has been suggested to be responsible for packaging specificity via binding to ATPase gp16, analogous to the DNA-binding small terminase subunit in phages with concatemeric DNA (Guo et al., 1987a). Phi29 packaging is unique in involving five or six molecules of virally-encoded structural RNA, pRNA (for *pro*head or packaging RNA) (Guo et al., 1987b; Chen and Guo, 1997; Ibarra et al., 2000; Simpson et al., 2000; Morais et al., 2001). The pRNA is probably bound to the portal complex (Xiao et al., 2005b), but

alternatively binding to the 5-fold vertex has been considered as well, at least in theory (Simpson et al., 2000; Guasch et al., 2002). pRNA is needed for binding of phi29 ATPase gp16 to the procapsid and for stimulation of the ATPase activity of gp16 (Grimes and Anderson, 1990; Lee and Guo, 2006). Initiation of phi29 packaging involves a sequence-independent inter-action of the terminal protein with downstream DNA to form a lariat structure (Grimes and Anderson, 1997). Multiple copies of packaging ATPase gp16 bind to the lariat junction and allow the introduction of supercoils to the structure (Grimes and Anderson, 1997), followed by binding of the lariat loop structure to the connector in the prohead (Anderson and Grimes, 2005). Exactly how the DNA is translocated into the prohead is not known, but a ratchet mechanism involving passive rotation of the portal, and specific interactions between pRNA-ATPase, connector and DNA, has been proposed (Simpson et al., 2000). Both ATPase and pRNA dissociate from the particle after packaging (Guo et al., 1987b; Chen and Guo, 1997; Peterson et al., 2001).

1.4.1.3 Accessory proteins

In addition to terminases, packaging ATPases and portal proteins, several other proteins have been discovered that are involved in DNA packaging. Some of these proteins may act by sealing or 'plugging' the portal of packaged capsids and preventing leakage of the packaged DNA. Examples of these include the bacteriophage SPP1 head completion proteins gp15 and gp16 that bind to the portal of DNA-filled SPP1 virions (Lurz et al., 2001), phage lambda gpW and gpFII proteins (Perucchetti et al., 1988; Murialdo et al., 2003), and possibly the phage P22 head completion proteins gp4, gp10 and gp26 (Strauss and King, 1984; Andrews et al., 2005). Some proteins are more general in function, and probably act by stabilising the mature packaged virions by binding elsewhere on the capsid, outside the packaging machinery, as the HSV-1 protein UL25 has been suggested to operate (Stow, 2001; Thurlow et al., 2006).

Other proteins may be involved in the actual packaging process together with the terminase and portal. The HSV-1 UL33 protein has been shown to interact with the packaging machinery via binding to the UL28 terminase subunit (Beard et al., 2002; Yang and Baines, 2006). The bacteriophage lambda gpFI protein has been implicated in DNA packaging and cleavage of concatemers, although in some conditions, gpFI is not strictly necessary (Benchimol et al., 1978; Benchimol et al., 1982). GpFI is needed for the initial cos-cleavage of lambda DNA, possibly via promoting binding of the DNA-terminase complex to the prohead (Davidson and Gold, 1987; Becker et al., 1988; Sippy and Feiss, 2004).

Host proteins may also be involved, for example the *E. coli* IHF protein has been reported to bind to lambda DNA cooperatively with the gpNu1 small terminase subunit and cause a bend in the DNA (Kosturko et al., 1989; Ortega and Catalano, 2006). IHF binding to DNA has been suggested to promote the assembly of an active DNA translocating complex essential for cos-cleavage and DNA packaging (Maluf et al., 2005).

1.4.1.4 Citius, altius fortius – Forces in DNA packaging

Bacteriophage packaging machineries are among the most powerful molecular motors found to date. The forces involved in bacteriophage phi29 DNA packaging have been studied by single molecule experiments in an optical trap (Smith et al., 2001). It was found that the last segments of DNA are packaged against an internal force of 50 pN, (equivalent to a pressure of 6 MPa), and the maximum stall force of the phi29 packaging machinery was measured to be approximately 70 pN (Smith et al., 2001)!

It has been estimated that in bacteriophage phi29, an average of 1 molecule of ATP per each 2 bp packaged is needed (Guo et al., 1987a), and a similar value of 1.8 bp/molecule of ATP has been reported for bacteriophage T3 (Morita et al., 1993). A much higher ATP consumption (6-10 ATP molecules per bp of DNA) has been reported for bacteriophage lambda, although this may contain some background ATPase activity by surplus terminase molecules not taking part in the actual DNA packaging process (Hwang and Feiss, 1995; Yang and Catalano, 2003). DNA packaging is very fast: estimates for the rate of DNA translocation vary between different bacteriophages, for example in bacteriophage T3, a constant rate of 5.7 kbp/min (at 20 °C) and (22 kbp/min (at 30 °C) has been reported (Shibata et al., 1987), whereas in phage SPP1 an average rate higher than 350 bp/s (21 kb/min) has been suggested (Oliveira et al., 2005).

1.4.2 Conformational changes in virus capsids in relation to DNA packaging

DsDNA packaging in icosahedral dsDNA viruses is typically accompanied by major changes in both individual capsid protein subunits and the overall capsid structure. These changes are usually irreversible and lead to removal of possible scaffolding proteins, an increase in the internal volume of the capsid and progression from a meta-stabile procapsid to a more stable mature capsid structure that is better able to protect the genome.

Studies on bacteriophages HK97, T7, T4, lambda and P22 (Laemmli et al., 1976; Steven et al., 1976; Earnshaw et al., 1979; Steven et al., 1992; Dokland and Murialdo, 1993; Prasad et al., 1993; Conway et al., 1995; Agirrezabala et al., 2005a) have shown that during capsid maturation, spherical procapsids containing scaffolding proteins expand into larger, more angular capsids with thinner walls. The increase in inner volume has been estimated in many bacteriophages to be around 50 % or even more (Earnshaw et al., 1979; Dokland and Murialdo, 1993; Agirrezabala et al., 2005a). Often, maturation involves changes in the relative position of capsid proteins and conformational changes in capsid protein subunits or even refolding of parts of the major capsid protein, as in the case of P22 (Jiang et al., 2003) and HK97 (Conway et al., 2001; Wikoff et al., 2006). In procapsids, the hexameric capsomers are often somewhat skewed or elongated in appearance, not presenting proper 6-fold symmetry, but in the mature capsid, the hexamers adopt a more symmetrical form (Dokland and Murialdo, 1993; Prasad et al., 1993; Conway et al., 1995; Zhang et al., 2000; Conway et al., 2001; Cerritelli et al., 2003; Jiang et al., 2003; Agirrezabala et al., 2005a). Procapsids may also contain relatively large holes in their walls, which are sealed in the mature virion and have been suggested to allow the escape of scaffolding protein from the procapsid, as is seen for example in bacteriophage P22 (Prasad et al., 1993). In bacteriophage T7, DNA packaging and maturation also involve re-arrangement of the unique core complex consisting of proteins needed for infection (Agirrezabala et al., 2005a). In many viruses, such as bacteriophages T4 and HK97, maturation expansion involves proteolytic cleavage of one or more capsid protein types, possibly creating a metastabile intermediate helping to overcome an energy barrier and facilitating the conformational changes needed to achieve the mature structure (Laemmli, 1970; Laemmli et al., 1976; Steven et al., 1992; Conway et al., 1995).

Maturation expansion may also reveal new epitopes on the capsid surface (Steven et al., 1991), and give the capsid the ability to bind additional proteins, possibly conferring further stability to the structure, as in the case of the bacteriophage T4 Soc protein (Steven et al., 1992; Iwasaki et al., 2000). In bacteriophage T7, packaging-related changes in the position and structure of the core complex have been suggested to push the terminase complex into a position where it is more accessible to tail components, allowing formation of the connector-tail structure (Agirrezabala et al., 2005a). In bacteriophage HK97, the combination of proteolytic cleavage and the subsequent capsid expansion is suggested to create a micro-environment necessary for the autocatalytic formation of new cross-linked peptide bonds (Conway et al., 1995). In bacteriophage phi29 and HSV-1, no capsid expansion occurs and the inner volume of the capsid probably does not change significantly, although angularisation of the structure occurs to give the mature capsid structure (Bjornsti et al., 1983; Newcomb et al., 1996; Trus et al., 1996).

In many (but not all) bacteriophages, the increase in capsid volume is clearly necessary to accommodate the full genome, as has been estimated for T4: the 169 kbp genome would not fit into the unexpanded procapsid, and only after expansion and a 50% increase in the capsid volume can the capsid accommodate the whole genome (Earnshaw and Casjens, 1980). However, it is still unclear exactly how DNA packaging, removal of scaffolding proteins and capsid expansion or other structural

changes are coupled to each other. Do scaffolding removal and capsid expansion precede packaging and enable it, or are they triggered by packaging?

As studied by *in vitro* packaging systems of lambda, T3, T7, and P22, unexpanded proheads containing scaffolding protein are the substrate for packaging enzymes (Hohn and Hohn, 1974; Poteete et al., 1979; Masker and Serwer, 1982; Shibata et al., 1987). In the case of bacteriophage lambda, it has been shown that particles are still packageable after scaffolding core exit (Hendrix and Casjens, 1975)**,** but not after expansion (Earnshaw and Casjens, 1980). The case for bacteriophage T4 has been especially confusing, as it was shown by Rao and Black (1985) that expanded particles, from which scaffolding had been removed, could be packaged *in vitro*, a phenomenon not seen in other bacteriophages. Mature, expanded T4 proheads have also been shown to be more active in packaging plasmid DNA *in vitro* than unexpanded proheads (Black and Peng, 2006). However, *in vivo* this does not seem to be the case: only scaffolding containing unexpanded proheads were shown to be used as substrates for packaging, whereas empty, expanded particles never proceed to mature virions *in vivo* (Jardine and Coombs, 1998; Jardine et al., 1998). In T4, several maturation intermediates have been detected: starting from the scaffolding-containing procapsid the maturation progresses via removal of the scaffolding to form empty, unexpanded proheads, then an unexpanded particle where packaging has been initiated, and after around 8 % of the genome has been packaged, expansion occurs and DNA packaging continues until the whole genome is packaged and the packaging process is completed (Jardine and Coombs, 1998). Similarly, in bacteriophage T3, capsid expansion has been reported to occur when approximately 25 % of the genome length has been packaged (Shibata et al., 1987). In bacteriophage phi29, even though the capsid volume does not significantly change, rearrangement of the capsomers and formation of the more angular mature capsid structure occur after packaging less than 10% of the genome (Bjornsti et al., 1983). In bacteriophage P4, it has been shown that proteolytic cleavage of the capsid protein by trypsin is alone, without the presence of DNA, enough to drive capsid expansion *in vitro* to an expanded, but not quite mature state (Wang et al., 2003). As it is not known which occurs first *in vivo*, packaging or cleavage, the it has been suggested that the cleavage of the capsid protein could be needed to enable the expansion, instead of actually triggering it, and that DNA packaging would be required for full maturation (Wang et al., 2003).

It seems obvious that to accommodate the genome, scaffolding proteins need to be removed and completion of expansion needs to precede the completion of packaging, if not its initiation. Several models have been suggested as to how expansion is triggered: first, the incoming DNA could cause a structural change in the portal and the neighbouring capsid protein subunits which would then trigger a change in the neighbouring subunits, and the structural changes would proceed as a wave from that one point to cover the whole capsid, as suggested by Prasad et al. (1993). In the second model, expansion could be triggered at multiple sites and would be due to the physical interaction of the DNA with the negatively charged inner surface of the capsid, as proposed for P22 and HK97 (Parker and Prevelige, 1998; Conway et al., 2001). Involvement of the portal is somewhat contrasted by the fact that in for example HK97 and P4, even though DNA packaging requires the portal structure, capsid expansion and cross-linking (in the case of HK97) can be induced in the absence of DNA and/or portal (Conway et al., 1995; Duda et al., 1995a; Wang et al., 2003). However, the absence of a portal could be considered unnatural and the results may not reflect packaging *in vivo* very well.

1.4.3 Structure of the packaged DNA

As stated earlier, the purpose of DNA packaging is not just to enclose the DNA within the capsid shell, but to do this in a way that after delivery to a new host cell, the DNA is topologically ordered in such a way that it can easily be used for a new round of infection. It should also be noted that due to its charged phosphate backbone, DNA is self-repulsive, in addition to being relatively stiff, not willingly bending back on itself over very short distances. Exactly how double-stranded DNA is ordered in viral capsids, except that it is mostly in B-form (Lepault et al., 1987; Aubrey et al., 1992; Tuma et al., 1996; Overman et al., 1998), has been the topic of debate for decades.

Several models for the organisation of DNA within the capsid (see Fig. 6) have been proposed, some of which have more experimental evidence to back them than others (discussed in Jardine and Anderson, 2005): A) the liquid crystalline model, B) the folded toroid model, C) the spiral fold model, and D) the solenoid model.

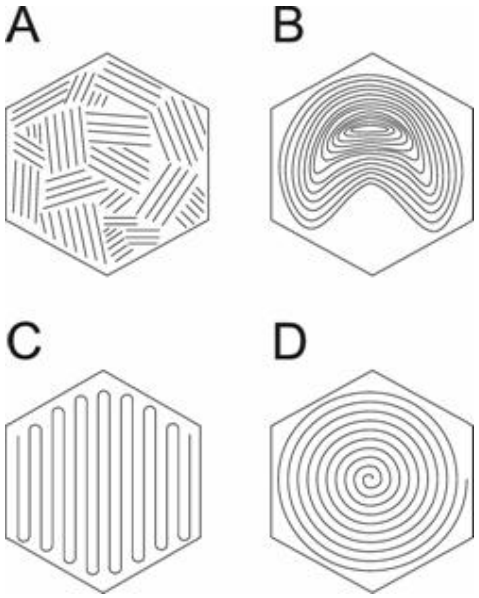

Figure 6. Models for DNA structure in the virion.
A) The liquid crystal, B) the collapsed toroid, C) the spiral fold, and D) the solenoid model. (Redrawn from Jardine and Anderson, 2005.)

In the liquid crystalline model (A), the DNA within the capsid would form multiple, relatively small, tightly packed, hexagonally ordered crystalline arrays, which are separated by short stretches of disordered DNA (Lepault et al., 1987). However, apart from the studies of Lepault et al. (1987) on bacteriophage T4, there is not much experimental evidence to support this model. In model B), the folded toroid (Hud, 1995), the DNA would be hexagonally packed to form a large donut-shaped structure with a hole in the middle. During the packaging process, this structure would then collapse into a folded structure. Unfortunately, although this model may not be totally inconsistent with the observed structure of DNA in mature virions, it doesn't explain in a satisfactory way how such a structure could be achieved, as the diameter of the torus that would collapse into the folded form, fitting the capsid, is clearly larger than the space available in the prohead (Jardine and Anderson, 2005). In the spiral fold model (C) suggested by Black et al. (1985), hexagonally packed DNA forms bundles of straight rods winding up and down along the long axis of the prohead. This model implies that DNA would be bent back on itself, forming 180° turns, which does not seem very likely, taking to account the stiffness of the DNA backbone. Interestingly though, there are several papers presenting experimental evidence (although indirect) supporting this model. In the last and oldest model, the solenoid (D), the DNA would be ordered in the form of a solenoid, or layered spool (reviewed in Jardine and Anderson, 2005). This model takes to account both the self-repulsiveness and stiffness of DNA. A mechanism has also been suggested as to how such a structure could be achieved: In the early phases of DNA packaging, the DNA would form loose loops within the proheads, following the longest path around the inner surface of the prohead, avoiding sharp bends. As more DNA enters, concentric shells would start forming, DNA repulsion determining

strand separation. Dynamic computer modelling by Kindt and co-workers (Kindt et al., 2001) supports this model, and the formation of concentric DNA layers progressing from the outside to the inside of the solenoid. It has further been suggested that the formation of a solenoid would require either occasional changes in the direction of spooling (Serwer, 1986) or axial rotation of the translocation machinery (Jardine and Anderson, 2005), the latter which is already a component of several models for the mechanism of DNA packaging.

The solenoid model has now gained more experimental evidence to back it. In cryo-EM structures of bacteriophages P22, T7 and epsilon15, from three to six concentric layers of hexagonally packed DNA, arranged as a coaxial spool along the vertical axis (along the middle of the portal) of the capsid, can be observed (Cerritelli et al., 1997; Agirrezabala et al., 2005a; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006). In T7, the spools of DNA may be wrapped around the internal core structure (Agirrezabala et al., 2005a). The spacing of the concentric DNA layers is approximately 25 Å (center-to-center), and in all cases, the outer layers are better organised than the inner ones (which are often not visible in the structures), suggesting that the outer layers are formed first (Cerritelli et al., 1997; Agirrezabala et al., 2005a; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006). Similarly, in bacteriophage T4, six concentric layers of hexagonally packed DNA, separated by 25 Å, can be seen (Fokine et al., 2004). In epsilon15 and T7, a tubular density is seen inside the portal and/or core, attributed to the terminal segment of DNA, ready for injection (Agirrezabala et al., 2005a; Jiang et al., 2006). In P22, a similar tubular density is seen, and in one study suggested to be DNA (Chang et al., 2006), but interpreted by others not to be DNA but protein (Lander et al., 2006). In the T4 tail structure, a similar density, attributed either to a tape-measure protein or to DNA, has been observed (Leiman et al., 2004).

The internal pressure created by the tight packaging of DNA within the capsid has been proposed to be important for DNA ejection (Kindt et al., 2001; Smith et al., 2001; Tzlil et al., 2003; Purohit et al., 2005), although the pressure alone may not determine entry of the DNA into the cell (Molineux, 2006). In addition to possibly participating in DNA ejection, the high internal pressure puts demands on the head completion proteins or other proteins involved in stabilising the portal vertex and the overall capsid structure after packaging, as ejection should occur only in a controlled manner when a suitable host is met and the cell wall penetrated. Ejection forces and mechanisms are discussed in more detail in relation to bacteriophage entry in section 1.7.3.

1.4.4 Genome packaging in eukaryotic icosahedral dsDNA viruses

As described above, the packaging machineries of herpesviruses are very similar to those of tailed bacteriophages. Similar mechanisms involving preformed particles may also be in use in other eukaryotic dsDNA viruses: empty particles devoid of DNA have been reported for both wt and mutant adenovirus infection (Edvardsson et al., 1976; D'Halluin et al., 1978a; D'Halluin et al., 1978b; Edvardsson et al., 1978), and a mechanism similar to that used by many bacteriophages has been suggested (Ostapchuk and Hearing, 2005). However, a mechanism where the capsid would assemble around the DNA in a coassembly reaction has also been suggested (Finnen et al., 2001; Zhang and Imperiale, 2003), so there is still an on-going dispute whether adenovirus actually uses preformed capsids or not. Regions in the adenovirus genome necessary for packaging have been identified, and interestingly, the terminal proteins and inverted terminal repeats (ITRs) were found to be non-essential for packaging (Ostapchuk and Hearing, 2003). Several packaging-related adenovirus proteins have been reported, among them the IVa2 and L1 52/55 proteins, which bind to packaging sequences on the DNA, and are needed for capsid assembly and DNA encapsidation, respectively, and the adenovirus L4 22 kDa protein, which is needed for efficient DNA encapsidation (Zhang et al., 2001; Zhang and Imperiale, 2003; Perez-Romero et al., 2005; Ostapchuk et al., 2006; Perez-Romero et al., 2006).

Although vaccinia virus (VV) particles are not icosahedrally shaped, spherical assembly intermediates (called IVs, immature virions) that are covered in a 'honey-comb' lattice formed of trimers of VV protein D13, reminescent of the bacteriophage external scaffolding proteins and subsequently lost upon virus maturation into brick-shaped particles, have been identified (Heuser, 2005; Szajner et al., 2005). A putative packaging ATPase has also been reported in vaccinia: without the A32 protein, only empty spherical particles devoid of DNA are produced (Koonin et al., 1993; Cassetti et al., 1998). Interestingly, also the large icosahedral dsDNA mimivirus has been reported to display at one of its vertices a large portal-like structure, possibly related to DNA packaging and/or injection (Xiao et al., 2005a).

1.4.5 Genome packaging in ssDNA bacteriophages

The icosahedral ssDNA containing viruses of the *Microviridae* have also been shown to first form procapsids which are subsequently packaged with DNA. However, no portal-like structures have been identified for microviruses. The ssDNA of phiX174 is packaged together with the highly positively charged DNA-binding protein J, which is also a component of the mature virion (Shank et al., 1977; Hamatake et al., 1985; Bernal et al., 2004). DNA packaging is linked to ssDNA synthesis, and a DNA packaging initiation and ssDNA synthesis complex, which is bound to the procapsid, has been identified (the 50S preinitiation complex) (Fujisawa and Hayashi, 1976; Fujisawa and Hayashi, 1977a; Aoyama et al., 1981; Aoyama and Hayashi, 1986; Ekechukwu et al., 1995). The DNA-J complex is assumed to enter the capsid through pores at the three-fold axes of the capsid (Ilag et al., 1995; Bernal et al., 2003; Uchiyama and Fane, 2005). In relation to DNA and protein J packaging, internal scaffolding protein B is extruded from the capsid, and a major structural change occurs in capsid protein F, to yield the so-called provirion (Ilag et al., 1995; Dokland et al., 1999). After completed packaging, the external scaffolding protein D is also dissociated from the provirion. (Ilag et al., 1995; Bernal et al., 2003).

1.4.6 Packaging of dsRNA genomes into preformed particles

DsRNA viruses such as the cystoviruses, and possibly also the rota- and reoviruses, similarly form empty particles which are subsequently packaged. However, the synthesis and packaging of dsRNA genomes presents some unique problems, not encountered by other types of viruses: the presence of dsRNA in a cell is an 'unnatural' phenomenon, and eukaryotic cells recognise dsRNA as something foreign and potentially dangerous, and quickly respond, attempting to eliminate the intruder RNA. For this reason, the genomes of dsRNA viruses are never exposed to the cell but always kept inside a viral core particle. For example, in cystovirus phi6, the core particle functions first as transcription machinery, spinning out mRNA for protein production (Gottlieb et al., 1990). New empty core particles are then assembled, followed by packaging of the (+)strand ssRNA (Gottlieb et al., 1990). Only inside the core particles is the ssRNA then replicated by an RNA-dependent RNA polymerase to dsRNA (Gottlieb et al., 1990). The genomes of dsRNA viruses are often segmented, for example bacteriophage phi6 of the *Cystoviridae* family has three genome segments. This is an additional challenge, as it has to be ensured that exactly one copy of each segment is packaged.

The packaging of bacteriophage phi6 has been studied in detail and a defined *in vitro* assembly and packaging system exists (Poranen et al., 2001). First, the procapsid, or as it is called in this case, the polymerase complex (PC), is assembled from four different proteins: protein P1 forming the main core structure, RNA-dependent RNA polymerase P2, packaging NTPase P4 and a additional packaging factor P7 (Olkkonen and Bamford, 1987; Gottlieb et al., 1992; Juuti and Bamford, 1997; Makeyev and Bamford, 2000; Huiskonen et al., 2006). Packaging NTPase P4 forms hexameric rings at the vertices (de Haas et al., 1999), through which the ssRNA is packaged, concomitantly with minus strand synthesis inside the particle by the P2 polymerase, and during transcription, the same P4 rings act as passive conduits for exit of mRNA from the particles (Pirttimaa et al., 2002; Kainov et al., 2004). Although P4 is present at all vertices, it has been proposed that only one of the P4 hexamers would be used for packaging, similar to the portal of tailed dsDNA phages, but all the rest of the P4 hexamers would be used for transcription (Pirttimaa et al., 2002). The three RNA segments: small (s), medium (m) and large (l), are packaged in a sequential manner (Frilander and Bamford, 1995; Mindich, 1999). A model has been proposed for phi6 packaging where the empty procapsid would contain a binding site for the s segment and subsequent packaging of s would lead to a conformational change in the PC that create a binding site for the m segment, the packaging of which would in turn create a binding site for the l segment (Frilander and Bamford, 1995; Mindich, 2004). Cryo-EM investigations on the phi6 nucleocapsid (the PC with a surrounding P8 protein layer) have now revealed structural information supporting this model (Huiskonen et al., 2006).

1.5 The bacterial cell wall

To initiate productive infection, bacteriophages need to both recognise and bind their host cells and to then cross the complex barrier formed by the cell wall. At the end of the virus life cycle, the cell wall needs to be crossed again, this time by the progeny virions.

1.5.1 Composition of the cell wall

The cytoplasm of eubacterial cells is surrounded by a lipid bilayer, the cytoplasmic, or *inner*, membrane (CM). The cytoplasmic membrane in gram-negative bacteria is very similar to that in gram-positive ones: it consists mainly of the phospholipids phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG). The cytoplasmic membrane is further enclosed within a murein sacculus, a net-like structure, or exoskeleton, composed of peptidoglycan, providing protection against osmotic pressure and defining the cell shape (except in *Mycoplasma* species and a few other exceptions). In gram-negative bacteria the peptidoglycan layer is further surrounded by a second lipid bilayer, the outer membrane (OM). The space between the CM and OM, occupied mainly by peptidoglycan, is called the periplasmic space. In gram-positive cells, there is no OM, but the peptidoglycan layer is significantly thicker. Schematic pictures of the structures of gram-positive and gram-negative cell walls are presented in Fig.7a. Additional components, such as slime or a capsule layer, pili, fimbriae or flagella may be present in some bacterial strains.

The terms cell wall and cell envelope are sometimes used somewhat confusingly: 'cell wall' and 'cell envelope' may be used interchangeably to mean the whole structure surrounding the cytoplasm of a bacterial cell, or alternatively, 'cell wall' may be used to refer only to the outer peptidoglycan layer of gram-positive cells, and 'cell envelope' to refer only to the membranous part of the structure. Here, the term 'cell wall' will be used to refer to the whole structure, including the CM, the peptidoglycan layer, and in the case of gram-negative bacteria, also the OM. The term 'envelope' will be reserved for enveloped viruses, when applicable.

1.5.1.1 The murein sacculus

Peptidoglycan (also called murein) is a huge heteropolymer, formed of a glycan backbone crosslinked by peptides (for recent reviews, see (Delcour et al., 1999) and (Höltje, 1998)). The glycan backbone is formed of $\beta(1\rightarrow4)$-linked disaccharide subunits composed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), with short peptide chains linked to the carboxyl group of the NAM subunits (see Fig. 7b). The long NAM-NAG backbones are assumed to be located parallel (horizontal) to the CM and are joined together by cross-links formed by transpeptidation reactions between the peptides (Vollmer and Höltje, 2004), although a structure of strands perpendicular to the CM has also been suggested (Dmitriev et al., 1999). Cross-linking of the peptides leads to formation of a huge, cross-linked network of peptidoglycan, essentially a 'sac', or *exoskeleton*, functioning also as a molecular sieve, around the CM.

The thickness of the murein layer has been the subject of much debate, and estimations on how many peptidoglycan layers are present have varied. Newer suggestions vary from a combination of only one layer of peptidoglycan (thickness of the peptidoglycan layer is 2.5 nm) in some areas, and a triple layer of peptidoglycan (7.5 nm) in others, as shown by small-angle neutron scattering

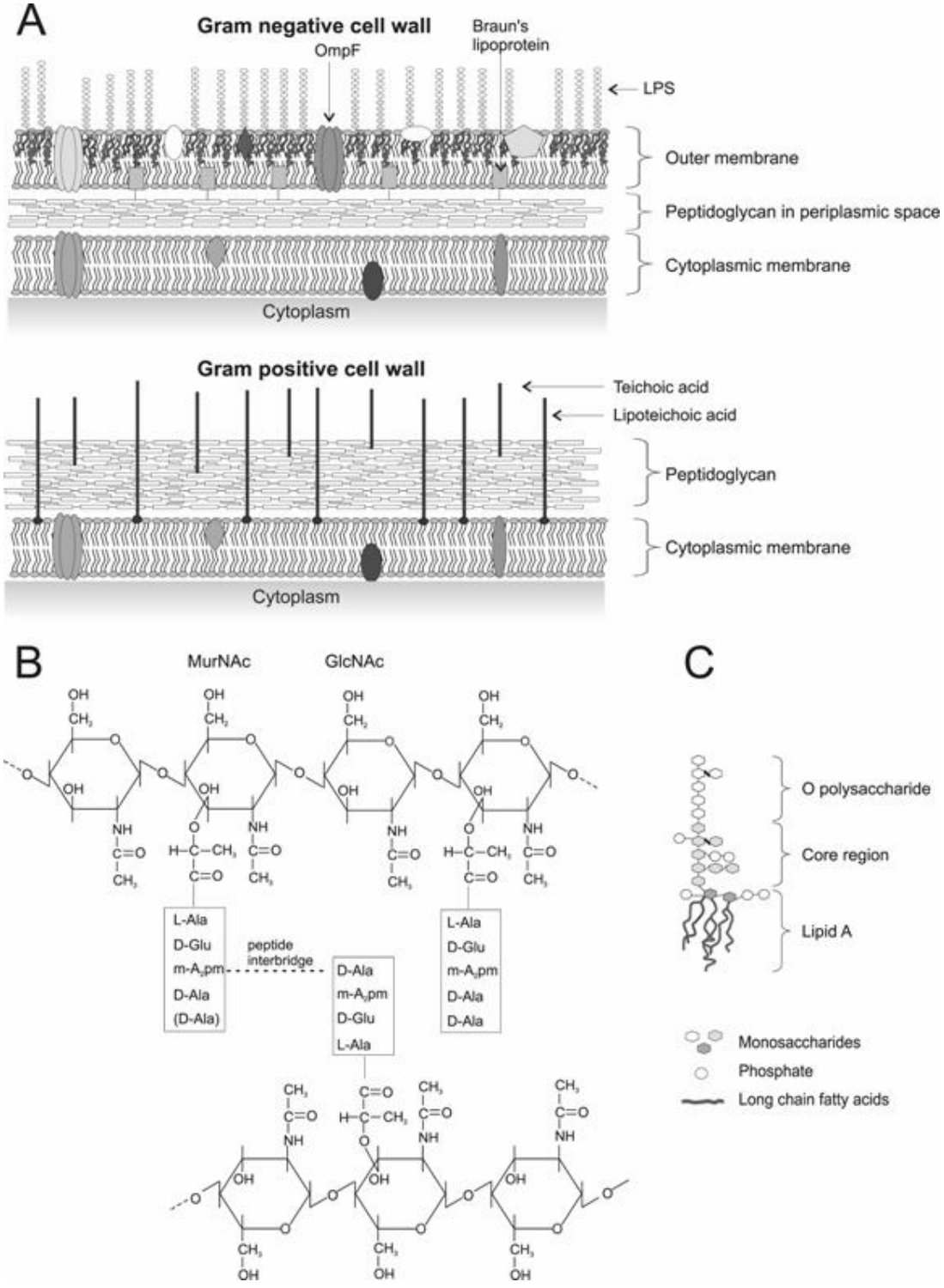

Figure 7. The bacterial cell wall.
A) Structure of the gram-negative and gram-positive bacterial cell walls, B) structure of peptidoglycan, and C) structure of lipopolysaccharide (LPS).

(Labischinski et al., 1991), or probably more than one layer, as studied by atomic force microscopy (AFM) (observed thickness 6 nm) and cryo-electron microscopy (thickness 6.35 nm) (Yao et al., 1999; Matias et al., 2003). The murein sacculi of gram-positive bacteria (reviewed for example in Schäffer and Messner, 2005) are thicker and generally more diverse in their chemical composition than the gram-negative murein sacculi and typically contain large amounts of teichoic acids and lipoteichoic acids, in addition to other cell wall polysaccharides and proteins.

Murein biosynthesis (Höltje, 1998; Delcour et al., 1999) is a complex process, involving many sequential enzymatic steps. It can be divided in two main parts: I formation of the disaccharide peptide building blocks, and II, polymerisation reactions, each stage consisting of multiple enzymatic reactions. To insert new glycan units into the cell wall, existing bonds in the peptidoglycan network need to be broken. For this purpose, cells have specific murein degrading enzymes, attacking the various different types of bonds in the peptidoglycan. Bacteriophages use similar enzymes to gain entry to cells, or to lyse cells at the end of their life cycle; these will be discussed in more detail in section 1.6 and 1.7.

1.5.1.2 The outer membrane and lipopolysaccharides of gram-negative bacteria

The outer membrane (OM) surrounding the peptidoglycan layer of gram-negative bacteria is a complex structure forming a semi-permeable, selective barrier. The OM is an asymmetric lipid bilayer, the inner leaflet being comprised primarily of phospholipids (mainly phosphatidyl-ethanolamine, phosphatidylglycerol and cardiolipin) and the outer leaflet of lipopolysaccharide, LPS. (For a review on OM structure, see Nikaido, 2003).

LPS is an amphipathic molecule composed of three parts: I) lipid, II) a highly charged, phosphorylated core polysaccharide, and III) a highly variable O-antigen (O-specific side chain) protruding from the cell surface (Wilkinson, 1996). LPS from which the O-antigen is absent, is termed R (rough), as opposed to the normal S type (smooth). For a schematic picture of LPS structure, see Fig.7c. LPS is anchored to the outer leaflet of the OM mainly via the fatty acyl chains of the Lipid A portion, and additional stabilisation is offered by interactions with proteins and divalent cations in the OM. LPS provides an efficient permeability barrier for the diffusion of lipophilic compounds.

The OM contains a high number of hydrophilic channels formed by porins, transmembrane β-barrel proteins functioning in the non-specific transport of hydrophilic nutrients and other small molecule compounds, protein channels functioning in the transport of specific molecules such as maltose or sucrose, and proteins involved in the secretion of bacterial proteins (Nikaido, 2003). Many of the OM proteins and LPS components are used as receptors by bacteriophages, as will be reviewed in section 1.7.1.

The inner leaflet of the OM is connected to the peptidoglycan layer by covalent bonds between the peptidoglycan and a small but abundant lipoprotein, Braun's lipoprotein (also called major lipoprotein) (Suzuki et al., 1978). Another important group of stabilising lipoproteins, called peptidoglycan-associated lipoproteins (PALs), are also found in the inner leaflet (Mizuno, 1979; Lazzaroni and Portalier, 1992).

1.6 Lysis

Infected cells do not merely burst apart when sufficient amounts of phage particles have accumulated in the cytoplasm, instead, lysis is a regulated process achieved with the help of specific enzymes in a timely manner.

1.6.1 Multi-component holin-endolysin systems

Cell lysis by dsDNA bacteriophages is an exactly timed event, as lysis should neither happen too early, before a sufficient amount of progeny virions have been produced, nor should it be delayed for too long, preventing the progeny virions from finding new host cells and multiplying. Optimally, lysis timing should be adjustable and take into account environmental factors such as the nutritional status of the infected cell, availability of new susceptible host cells and competition by other phages. All dsDNA phages seem to employ a multi-component cell lysis system, consisting of a minimum of two different types of lysis proteins: an endolysin (often called a lysozyme) and a holin, but often including several other regulatory components. (Reviewed in Young and Wang, 2005).

Endolysins are the enzymes responsible for destruction of the cell wall. They are peptidoglycan degrading enzymes that can be divided into four groups, based on which type of enzymatic cleavage they perform: muramidase (N-acetyl-muramidase or true lysozyme) hydrolyses the (1→4)-glycosidic bonds of the NAM-NAG sugar backbone of peptidoglycan, transglycosylase (endo-β-N-acetyl-glucosaminidase) acts on the same (1→4)-glycosidic bonds but the end product is a cyclic (1→6)-anhydro-N-acetylmuramic acid; amidases hydrolyse the amide bond between the sugar and peptide moieties of peptidoglycan and endopeptidase acts on peptide cross-bridges (Höltje, 1998). The bacteriophage endolysin genes are very similar to their bacterial counterparts, and have most probably been acquired by horizontal gene transfer from the host (Romero et al., 1990; Loessner et al., 1997)

In gram-negative bacteria, the peptidoglycan layer is enclosed between the inner and outer membranes, but in gram-positive bacteria, it is the outer-most component. For this reason, endolysins of phages infecting gram-positive bacteria are often modular: their enzymatic activity is located in the N-terminal domain of the protein, and the C-terminal domain is responsible for binding to the cell wall and preventing the enzymes from dissociating away from the peptidoglycan layer (García et al., 1990; Sanz et al., 1992; Croux et al., 1993; Loessner et al., 1997). Due to the modular nature of the proteins, the binding and cleavage domains of lytic enzymes are 'interchangeable' with other bacteriophage lysis proteins: domains may recombine separately during the course of evolution to form new combinations of binding and cleavage modules with new specificities or activities (Varea et al., 2004). The genes for the N and C terminal domains of two different phage lysins may also be joined together in the laboratory to produce functional, recombinant enzymes (Croux et al., 1993) and functional chimeric lysins have also been produced by combining phage and host lysin domains (Diaz et al., 1991). It is, however, possible to find more than one type of enzymatic activity in one lysin protein, in which case each binding and cleavage activity are located in a separate domain, as for example in the bacteriophages phi11 and B30 lysins, which possess endopeptidase and amidase or endopeptidase and glycosidase activities, respectively (Navarre et al., 1999; Pritchard et al., 2004). The C-terminal domains of endolysins are highly specific in their binding, which has raised hopes for using endolysins as specific antibacterial agents (Borysowski et al., 2006).

With the exception of certain endolysins containing sec-domains for secretion (São-José et al., 2000), endolysins cannot penetrate the cytoplasmic membrane and reach the peptidoglycan layer un-aided. For this purpose, there is a second component in the phage lysis system: holin. Holins are small membrane proteins that accumulate in the CM of the infected cell. During the final stages of infection, holins form 'holes' that permeabilise the cytoplasmic membrane and allow the endolysins access to the peptidoglycan layer. Holins have been found in phages with secreted endolysins as well, where their presence ensures more efficient lysis (Parreira et al., 1999; São-José et al., 2000). In such cases, the change in the state of the membrane caused by the holins has been suggested to activate the otherwise inactive endolysins (São-José et al., 2000).

Many bacteriophages have a third lysis factor, antiholin, which regulates the function of holin. Antiholins are usually, but not always, almost identical to the respective holin protein, with the exception of a few N-terminal residues, and produced by using alternate start codons for the same holin/anti-holin gene, the most studied example being that of the lambda S105 holin and S107 antiholin proteins (Bläsi et al., 1989; Bläsi et al., 1990). Antiholins act by binding to holin molecules to form holin-antiholin heterodimers that are prevented from inserting into the CM, the excess of holin over anti-holin depicting the amount of free holin available for insertion in the membrane and the rate of hole formation (Chang et al., 1995; Gründling et al., 2000). For example, in bacteriophage lambda absence of antiholin S107 shifts lysis to a slightly earlier time point, but an excess of antiholin can efficiently inhibit lysis (Raab et al., 1988).

Using alternate start codons for the same gene is not the only way to produce antiholins. In bacteriophage T4 a phenomenon called lysis-inhibition (LIN) has been observed in the case of attempted superinfection by another T4-type phage. It has been found that LIN is achieved by the T4 RI protein, which although unrelated to the T4 holin protein T in sequence, acts as an antiholin and binds holin T, effectively inhibiting lysis (Ramanculov and Young, 2001).

Holins, together with possible antiholins, are responsible for the timing of lysis, taking care that endolysins are activated rapidly and at a precisely defined time. It has been shown that the effect of holins is not gradual: no gradual changes in proton-motive force or ion gradients can be seen until suddenly, a total collapse and cell lysis occurs (Gründling et al., 2001). This has lead to the 'critical concentration model' where accumulation of holin in the membrane leads, after a certain concentration has been reached, to a local disruption of the membrane. This first lesion collapses membrane potential, which further triggers holin precipitation and membrane disruption (Gründling et al., 2001). Antiholins may thus act not only to ensure that lysis does not occur too early, but that until lysis, the cell is relatively healthy and capable of producing large amounts of virions, and that when lysis occurs, enough holin is present to ensure that it is rapid and efficient, and time is not wasted in an unproductive phase (Bläsi and Young, 1996; Gründling et al., 2000)). It has been proposed that certain changes in environmental conditions could lead to changes in the relative amount of holin and anti-holin and thus affect the timing of lysis (Gründling et al., 2000).

1.6.2 Amurins

Murein-degrading endolysins, let alone intricate multi-component holin-endolysin systems, have not been found in the small lytic ssDNA or ssRNA phages (the Levi-, Micro- and Allolevivirida e). It has only recently been shown that at least two viruses of this group encode proteins, termed amurins, that inhibit specific enzymatic steps in the murein biosynthesis pathway (Bernhardt et al., 2002). Amurins are thus essentially 'protein antibiotics'. Because amurins work by inhibiting cell wall synthesis, not by degrading existing structures, lysis of this type requires continued cell growth.

Bacteriophage ϕX174 protein E is a membrane protein, encoded by an alternate reading frame embedded in the reading frame of scaffolding protein D. Protein E acts on the MraY protein, an enzyme which catalyses the first membrane step of bacterial cell wall peptidoglycan synthesis producing so called 'lipid intermediate I' (Bernhardt et al., 2000; Bernhardt et al., 2001a). Bacteriophage Qβ protein A2 is similarly a single-component lysis protein, but in Qβ the lysis protein A2 performs multiple other functions, including RNA binding during capsid assembly, male pilus binding, and RNA 3' terminus protection (Karnik and Billeter, 1983; Winter and Gold, 1983). A2 also acts by inhibiting murein synthesis, but via action on MurA, an enzyme that catalyses the committed step in the murein biosynthesis pathway (Bernhardt et al., 2001b). Interestingly, another lysis protein, Rg, coded by a cryptic gene and causing much faster cell lysis than A2, was recently found in bacteriophage Qβ (Nishihara et al., 2004). It is speculated that this protein could be expressed at late stages of bacteriophage Qβ infection by a translational frame-shift mechanism, or that it may be only a non-active evolutionary remnant (Nishihara et al., 2004).

1.7 Bacteriophage entry

To gain access to a cell, and to be able to replicate, viruses must be able to both locate a suitable host organism and to transfer their genetic material into the cell. In the case of many eukaryotic viruses, the whole virus is internalised via different types of endocytosis mechanisms. In the case of most of the dsDNA bacteriophages, only the viral nucleic acid is transported into the cytoplasm, leaving the capsid protein outside the cell. In the case of dsDNA bacteriophage PM2, a mechanism involving coat dissociation and membrane fusion of the lipid core particle has also been suggested (Kivelä et al., 2004).

1.7.1 Binding to a suitable host cell

Bacteriophages use various different cell surface molecules as their receptors: in gram-negative bacteria, components of LPS may be used, as for example in the yersiniophage phiR1-37 (Kiljunen et al., 2005), or the phage may bind to outer membrane proteins such as the ferrichrome transporter FhuA, as in the case of bacteriophage T5 (Coulton, 1982). Other cell surface proteins or protein complexes, such as the outer membrane protein NfrA used by coliphage N4 (Kiino and Rothman-Denes, 1989), or the flagella or the pili, may also be used as receptors (Merino et al., 1990; Bennett and Rakonjac, 2006). Certain podoviruses have also been shown to bind to the polysaccharide K antigens in bacteria with capsules, in which case the phages need to carry enzymes able to digest the polysaccharide K layer (Scholl et al., 2001). In gram-positive cells, lipoteichoic or teichoic acids (Estrela et al., 1991; Wendlinger et al., 1996; Tran et al., 1999) or other non-protenacious components of the cell wall are often used as receptors (Valyasevi et al., 1990; Quiberoni et al., 2000; Geller et al., 2005), although for example bacteriophage SPP1 has been shown to use a protein receptor, YueB, in the *Bacillus subtilis* cell wall (São-José et al., 2004).

In tailed phages, the tail fibers located at the tip of the tail are used for receptor binding. In non-tailed icosahedral phages receptor-binding proteins are usually located at the capsid vertices, as in the dsDNA bacteriophage PM2 (Huiskonen et al., 2006), and in filamentous phages on one end of the virus particle (Goldsmith and Konigsberg, 1977). Many phages use two types of receptors. Binding to the primary receptor is often reversible and may cause a conformational change in the virion, leading to binding to the secondary receptor, which is commonly irreversible. For example, bacteriophage T4 has a complex tail structure formed of at least 14 different proteins (Leiman et al., 2004). In T4 infection, the long tail fibers (at least 3 fibers out of the 6 fibers) first bind reversibly to LPS components on the cell surface, which leads to a conformational change in the long tail fibers, and they adopt a so-called ‘down’ conformation (Leiman et al., 2004). This in turn brings the base-plate closer to the cell surface and allows the short tail fibers to irreversibly bind to the cell, and causes a major conformational change in the base-plate structure converting it from hexagonal to star-shaped and initiating tail sheath contraction (Leiman et al., 2004). Contraction of the tail sheath allows the tail tube, with its cell wall digesting enzymes, to protrude and access the cell surface.

1.7.2 Penetration of the cell wall

After binding to the cell, the phages need to be able to transport their DNA across the OM, through the highly viscous peptidoglycan layer, and across the CM into the cytoplasm. Similar to murein degradation to achieve lysis to liberate newly assembled virions, phages employ murein degrading enzymes to gain access to the cytoplasm of cells in the beginning of infection. In the case of cell entry, the enzymes need to be carried on the virions, and the damage to the murein layer is only local and does not kill the host bacteria.

Bacteriophage T5 uses the ferrichrome transporter protein FhuA as its receptor (Coulton, 1982). Addition of T5 to a preparation of liposomes with FhuA opens up high conductance ion-channels, and triggers the transfer of DNA into the liposomes, suggesting that FhuA also might provide the channel for T5 DNA transport across the OM (Plancon et al., 1997; Lambert et al., 1998). However, when T5 ejection into liposomes was studied by cryo-EM tomography, it was found that the tip of the T5 tail is able to traverse the lipid bilayer, and a conformational change, shortening and widening the tail occurs, probably providing a route for DNA translocation in the vicinity of FhuA (Böhm et al., 2001). Exactly how the T5 tail tube is able to penetrate the peptidoglycan layer is unclear, although it has been suggested that adsorption and DNA internalisation might occur preferentially in areas where the OM and the CM are in close contact, at so-called Bayer's patches (Bayer, 1968).

In bacteriophage T4, the tail tube crosses the whole cell wall providing a route for DNA entry. A complex of bacteriophage T4 tail-associated lysozyme gp5 and another phage protein, gp27, is responsible for penetration of the cell wall (Nakagawa et al., 1985; Kanamaru et al., 2002). The carboxy-terminal part of the gp5 protein of bacteriophage T4 is capable of puncturing the OM, whereas the middle domain harbours the lysozyme activity responsible for peptidoglycan digestion, and the N-terminal domain may be a part of the channel for DNA translocation (Kanamaru et al., 2002). Gp5 lysozyme is probably activated via cleavage by *E. coli* proteases after penetrating the OM (Kanamaru et al., 2005).

In bacteriophage T7 infection, the tail is not long enough to reach across the cell wall and another type of mechanism is used. Five viral proteins, gp14, gp15 and gp16, and the small gp6.7 and gp7.3 proteins, are injected from the virion at the initiation of infection (Kemp et al., 2005). After injection, gp14, gp15 and gp16 are suggested to form a channel across the cell wall for the DNA to traverse through, with gp14 residing in the OM (Molineux, 2001). Gp16 has a transglycosylase motif and can hydrolyse peptidoglycan *in vitro*, suggesting that gp16 is the protein responsible for digestion of the murein layer to allow T7 DNA access to the cytoplasm (Moak and Molineux, 2000; Moak and Molineux, 2004).

Similar murein hydrolysing enzymes have been identified in the virions of non-tailed phages as well: for example, a virion-associated endopeptidase enzyme has been identified in the icosahedral dsRNA bacteriophage phi6 (Caldentey and Bamford, 1992). Interestingly, in bacteriophage phi29, an endolysin activity has been associated with the gp3 genome terminal protein, the main role of which is in protein-primed DNA replication (Moak and Molineux, 2004).

1.7.3 Ejection of dsDNA

Tailed bacteriophages have traditionally been likened to hypodermic syringes. The high internal pressure created by the tight packaging of DNA within the capsid has been proposed to provide the energy necessary for ejection of the DNA from the capsid (Kindt et al., 2001; Smith et al., 2001; Tzlil et al., 2003; Purohit et al., 2005). However, ejection of the DNA is not just the reverse of DNA packaging, and in many bacteriophages, ejection has been shown to be an active process, involving both pushing and pulling forces. In addition to the energy stored in the packaged DNA, ATP may be needed, or the membrane potential may play a role in DNA ejection (Gonzalez-Huici et al., 2004; Kemp et al., 2004). In many phages transcription is required for complete internalisation of the phage DNA. The rate of phage DNA transfer into cells can be very high, as compared to other DNA transfer processes such as conjugation and natural transformation, for example a rate of 75000 bp/s has been estimated for bacteriophage T5 (Mangenot et al., 2005).

A pressure driven model for DNA ejection is supported by several findings: First of all, in bacteriophages lambda and T5, only contact with the respective receptor proteins is necessary for ejection of the whole genomes into liposomes or solution *in vitro* (Roessner et al., 1983; Lambert et al., 1998; Mangenot et al., 2005). Secondly, in lambda, ejection *in vitro* has been found to be suppressed by the addition of PEG to increase the external pressure, creating an opposing force for internal pressure driven ejection of DNA (Evilevitch et al., 2003; Evilevitch et al., 2005). Also, it has been shown that ejection of T5 DNA can be modulated by the addition of spermine, which reduces the pressure of the DNA inside the capsid and induces condensation of the released DNA, creating an additional pulling force (de Frutos et al., 2005). Additionally, in bacteriophage lambda, length of the packaged genome clearly affects the subsequent ejection of the genome into a new host cell: a shorter genome, causing a lower internal pressure inside the capsid being ejected with less force (Grayson et al., 2006). However, one of the observations that speaks against a solely pressure-driven mechanism is the fact that T5 ejection into cells actually continues even if the capsids are removed, provided that protein synthesis is allowed (Labedan and Legault-Demare, 1973).

In the case of T7, entry of the DNA is a much slower (5-40 bp/s) multi-step process where the first 850 bp of the genome enter the cell in a transcription-independent manner, via an enzymatic process controlled by the viral protein gp16, which is part of the channel structure for DNA ejection (Zavriev and Shemyakin, 1982; Garcia and Molineux, 1995; García and Molineux, 1996; Struthers-Schlinke et al., 2000; Kemp et al., 2004). Entry of the next 7 kb requires transcription by the cellular RNA polymerase, and the last 32 kb are drawn in by transcription by the T7 RNA polymerase, which is coded for by genes in the previously internalised section of DNA (Zavriev and Shemyakin, 1982; Garcia and Molineux, 1995). Transcription-dependent entry of bacteriophage DNA may provide a mechanism for protecting the DNA from restriction enzymes: the first T7 protein to be synthesised is a type I restriction enzyme inactivating-protein, which inactivates the cellular restriction enzyme before the recognition sites on the phage DNA enter the cell (Molineux, 2006).

In phi29, the genome enters in a two-step process: the first 65 % of the genome is pushed, probably by the internal capsid pressure, and the remainder of the DNA is pulled into the cell by a machinery that probably contains the viral gp17 protein (Gonzalez-Huici et al., 2004). The proton-motive force (PMF) has also been shown to be involved in pulling the DNA inside the cell (Gonzalez-Huici et al., 2006). I.e. phi29 DNA entry as such is not transcription-dependent, but transcription and translation are required for the production of gp17 and other viral proteins needed to 'pull' the DNA inside (Gonzalez-Huici et al., 2004).

1.8 Lysogeny

Infection of a host bacterium typically leads to synthesis of progeny virions, and ultimately, host cell lysis. However, many of the tailed dsDNA phages are able to exist in an alternative state called lysogeny, bacteriophage lambda being the classical example. In lysogeny, most of the detrimental bacteriophage genes are 'turned off', and progeny virions are not produced. Instead, phage DNA is replicated in concert with host chromosome replication and cell division. This stable relationship is either due to physical integration of the phage DNA into the host chromosome, as for example in bacteriophage lambda, or the phage DNA may exist as a separate plasmid, linear or circular, examples of which include bacteriophages P1, PY54 and N15 (Ikeda and Tomizawa, 1968; Rybchin and Svarchevsky, 1999; Hertwig et al., 2003). The latter mechanism, not involving integration, has also been called *pseudolysogeny*, although it may be just as stable or unstable as lysogeny involving integration. A bacteriophage that is in the state of lysogeny is called a prophage and phages that are capable of lysogeny are called temperate phages. However, it is unclear whether viruses should so strictly be divided into temperate and lytic phages. It has been shown that in addition to the lysogeny genes, temperate phages are not profoundly different from lytic phages, the closest relatives of lytic phages in many cases being temperate phages, and it has been suggested that many lytic phages could have arisen by deletions and rearrangements of the lysogeny genes of temperate phages (Mikkonen et al., 1996; Le Marrec et al., 1997; Ford et al., 1998; Lucchini et al., 1999).

To initiate integration, integrase enzymes (or transposases, as in the case of Mu) are needed. Alternatively, enzymes may be coded that cause circularisation of the genome in order for it to be maintained as a circular plasmid. The prophage may return to a lytic life style by a process called induction, either spontaneously or due to some environmental trigger. For the lysogenic state to persist, integration alone is not enough and the expression of lytic phase proteins needs to be inhibited. For this purpose, phages express specific repressor proteins during the lysogenic phase. The most studied example of repressor proteins is the bacteriophage lambda cI repressor. Expression of cI leads to inhibition of transcription from lambda early promoters, and subsequently, due to the lack of early gene products, inhibition of the expression of the late products as well. Bacteriophage lambda cI, as well as most other phage repressors, mimics the cellular LexA repressor protein, which is involved in regulating the SOS response in DNA damage. Both LexA and bacteriophage repressor proteins can undergo proteolysis mediated by binding to the RecA protein, leading to expression of DNA repair proteins and, in the case of the prophage, to induction (Little, 1984; Mustard and Little, 2000). Spontaneous induction of bacteriophages is assumed to happen in response to cellular DNA damage and activation of the SOS response by RecA and LexA.

Prophages are widely spread among bacterial genomes, probably because often the presence of a prophage can protect the host from further infection with the same type of phage, or provide some other advantage to the cell. In some cases, prophages are associated with increased virulence of the host bacterium, as in the Shiga toxin-converting bacteriophages in *E. coli* and *Shigella* species (Herold et al., 2004; Muniesa et al., 2004), or as in filamentous phage CTXPhi, which is responsible for cholera toxin production and the pathogenicity of *Vibrio cholerae* (Waldor and Mekalanos, 1996).

Bacterial genomes often contain defective phages or 'cryptic prophages'. These are phages that have lysogenised, but are in a state of mutational decay, and are therefore unable to perform a full cycle of virus replication, although some viral proteins may be produced. When the phages have been first inactivated, possibly only by a single mutation, further mutations may accumulate rapidly, as the selection pressure of being able to form a functional virion is removed. Defective phages can still be reactivated, if they recombine with another phage or prophage

that provides functional genes to replace the defective ones.

Prophages are important vehicles for horizontal gene transfer (Casjens, 2003). Imprecise excision of integrated phages from the host chromosome upon induction may lead to host sequences being packaged alongside the viral DNA, a phenomenon called specialised transduction. Integrated viral genomes may also undergo recombination with host sequences, producing new gene combinations. The role of temperate viruses in horizontal gene transfer will be discussed in more detail in section 1.9.

Some eukaryotic dsDNA viruses have been shown to undergo a similar process. The brown algae infecting phaeovirus ectocarpus siliculosis virus 1 (ESV-1) enters a latent phase in somatic cells by integrating into the host genome and multiplies only in cells of the reproductive organs (Delaroque et al., 1999). Most archaeal viruses are non-lytic and reside in the cell either as plasmids or are integrated into the genome, but are not actually lysogenic. Despite active virus production, the host cells are not lysed, and virus particles are extruded from the cell. For example, sulfolobus tengchongensis spindle-shaped virus 1 (STSV-1) is maintained as a stable circular episome in the cell and virions are produced without this leading to cell lysis (Xiang et al., 2005). However, very recently, ATV, a virus of the hyperthermophilic archaeal genus *Acidianus*, has been found to integrate into the genome of its host cell and to establish lysogeny, representing the first case of lysogeny in a crenarchaeal virus (Prangishvili et al., 2006).

1.9 Virus evolution and diversity

The diversity of viruses is almost too huge to understand. Viruses, as all replicating organisms, are prone to errors, and mutations accumulate in their genomes. With viruses, this process is faster because their life cycles are often shorter and large numbers of progeny are produced in an exponential fashion. In RNA viruses, mutations may accumulate even faster than in DNA viruses, as RNA-polymerases lack proof-reading mechanisms and have a much higher error-rates than most DNA-copying enzymes (Drake, 1991; Echols and Goodman, 1991; Drake, 1993). It has also been suggested that the mutation rate in ssDNA viruses may be higher than in dsDNA viruses, due to a higher rate of cytosine deamination in ssDNA (Frederico et al., 1990). In addition to accumulating point mutations, or insertions or deletions during the replication process, virus genomes may undergo recombination or in the case of multiple gene segments, reassortment.

The fast mutation rate of RNA viruses has lead to the quasispecies concept, first described for the phage Qβ (Domingo et al., 1978): for a given virus, each genome in an infected cell may be slightly different, and the virus cannot be defined by one sequence only, but the genome sequence is described as a population of sequences in dynamic equilibrium, clustering around a consensus or average sequence, and controlled by selection. Thus the evolution of RNA and DNA viruses differs both quantitatively and to some extent qualitatively, and only DNA virus evolution will be discussed in more detail below.

1.9.1 Horizontal gene transfer and mosaic genomes

As more and more phage genomes are sequenced, it is becoming evident that instead of clear hierarchical relationships, tailed dsDNA bacteriophages exhibit extensive mosaicism in their genomes, indicating wide-spread horizontal gene transfer, although elements of vertical evolution are also evident to variable extent in different virus groups (Hendrix et al., 1999; Juhala et al., 2000; Brüssow and Desiere, 2001; Pedulla et al., 2003). For example, some of the head genes of *Streptomyces* phage C31 are similar to the corresponding genes of HK97, and certain early C31 genes are similar to genes in bacteriophage L5, although HK97 and L5 share no direct sequence similarity, if only these two viruses were analysed (Hendrix et al., 1999). This has lead to the proposition that the tailed phages comprise a single large evolutionarily related group of viruses, for which horizontal evolution provides access to a common gene pool, although access to this gene pool would not be uniform for all members of the group (Hendrix et al., 1999).

Mosaicism of tailed bacteriophage genomes has actually been observed already several decades ago by heteroduplex mapping of lambdoid phage DNA and led to the 'modular theory' of phage evolution already a quarter of a century ago (Botstein, 1980). It was suggested that there might be special linker sequences, short stretches of conserved sequence, located between genes, facilitating recombination, or, alternatively, a site-specific recombination mechanism might operate that would explain the apparently 'more favoured' recombination sites in the genomes (Botstein, 1980). The accumulation of sequence data has since shone new light on the organisation of phage genomes and the modular theory has been refined. The current view is that non-homologous recombination actually occurs indiscriminately across the whole genome, but that most recombination events will lead to nonviable genomes, where reading frames are destructed, and genes are non-functional (either as such, or unable to interact with their counterparts), or the genome is too long or short to be packaged (Hendrix, 2002). Most non-destructive events will occur at boundary regions between genes, blocks of genes with

interacting protein products, or in some cases, between two domains of one protein-coding in gene. Thus, the apparent mosaic boundaries between genes or blocks of genes are due to the fact that only such recombination events lead to gene products producing viable bacteriophages. Homologous recombination is much more frequent than non-homologous, and homologous recombination has been proposed to play a significant part in distributing the new genes and combinations of genes initially acquired by non-homologous recombination throughout the population (Hendrix, 2002). It has now been shown that the idea of linker sequences was maybe not totally wrong either: short conserved sequences at boundaries of functional modules, suggested to be involved in homologous recombination, have actually been found in a group of lambdoid phages (Clark et al., 2001).

Theoretically, opportunities for horizontal genetic exchange between phages can arise by several different means: two viruses co-infecting the same host cell could directly recombine, or an infecting virus could recombine with a resident prophage, with either the prophage or the infecting phage acting as a gene donor, or third, prophages could recombine with each other. This would give temperate bacteriophages much more possibilities for horizontal gene exchange than strictly lytic phages, and would thus mean that more extensive mosaicism could be expected to be seen in temperate phage genomes (Lawrence et al., 2002). This indeed seems to be the case, as seen in the comparison of *Lactococcus* phages, where the sequences of temperate phages vary extensively but virulent phages exhibit much less genetic variation (Chopin et al., 2001). In large lytic T4-type phages, mosaicism is similarly absent: a core set of 24 genes encoding the replication and virion structural genes was found, of which the majority seemed to share a common evolutionary history, and only one core gene showed evidence of lateral gene transfer with the bacterial host (Filée et al., 2006; Nolan et al., 2006). Viruses of higher eukaryotes have even more limited opportunities for successful genetic exchange. Although many eukaryotic viruses may integrate into the host genome to form proviruses, any recombinant virus should be formed and released during the lifetime of the individual cells, as the provirus will not be passed on to the next generation unless it is integrated into the germ line; leading to less mosaicism (Lawrence et al., 2002).

Both their lytic life-style and the small size of their ssDNA genome are expected to limit the extent of horizontal gene transfer of viruses in the *Microviridae* family. Indeed, when genomes of microvirid phages were sequenced, they were found to form three distinct phylogenetic clades, with distinct genome sizes and gene contents, and with only two or three genes showing signs of horizontal gene transfer (Rokyta et al., 2006). Interestingly, such restrictions of horizontal transfer for genes coding for interacting proteins as seen in the tailed phages, leading to modules of several proteins, rather than individual proteins, being the units of transfer, were not apparent from the *Microviridae* genomes, and the genes exhibiting features of possible horizontal transfer were the major capsid gene F, spike protein G and the external scaffolding protein D (Rokyta et al., 2006).

Similarly, no apparent mosaicism can be seen in the *Tectiviridae* family (Saren et al., 2005), which will be discussed in more detail in section 1.10. Interestingly, the tectiviruses that infect gram-positive bacteria (of which at least some can establish lysogeny as plasmids) show more divergence in their sequences than the strictly lytic, PRD1-type tectiviruses that infect gram-negative bacteria (Saren et al., 2005).

It thus seems that vertical and horizontal evolution play different roles in the evolution of different types of viruses: in lambdoid phages and many other temperate phages horizontal evolution takes centre stage and almost totally masks the effects of vertical evolution, whereas in other, especially strictly lytic viruses, vertical evolution seems to be the main mechanism operating, and less evidence for horizontal evolution is seen. Whether this picture will be the final one, is yet unclear. Alternatively, it may also be a question of under-sampling of the virus

population. The diversity of phages is so great, and only such a small, and possibly biased, portion of that diversity has been sequenced, that accumulating new sequence data on bacteriophages and other viruses, as well as new methods for sequence analysis, may well totally change our view on virus evolution once more.

1.9.2 Acquisition of new genes

Temperate phages may acquire new genes by imprecise excision from the host genome, leading to bacterial genes flanking the prophage to be packaged (see also section 1.8). However, non-homologous recombination may also be a mechanism for the acquisition of totally new genes to the phage genome: comparative analysis of phage genomes has lead to the identification of genetic elements called *morons* (for *mor*e DNA), first described by Juhala et al. (2000). Morons are genes that are present in one phage but not in others of the group, neatly inserted between two genes. Morons are usually preceded by a functional promoter and followed by a terminator, and may provide the host with some advantage, which prevents deletion of the moron from the prophage. Morons clearly differ from the surrounding genome in their GC-content, suggesting a recent insertion via non-homologous recombination and possible later deletion of any 'extra' DNA inserted at the same time, instead of a mechanism where a common gene would have been deleted from all the other genomes of related viruses (Hendrix, 2002). It has even been proposed that bacteriophage evolution could have proceeded via a mechanism of 'moron accretion', starting from a single capsid protein gene, with step-wise addition of morons to the genome, each slightly increasing the fitness of the phage (Hendrix et al., 2000).

1.9.3 Taxonomical problems due to genetic mosaicism

Mosaicism leads to significant problems in phage taxonomy, as the supposed relationships between viruses will differ according to the genes chosen as a basis of classification. A new system has been proposed by Laurence et al. (2002), taking to account the mosaicity of viral genomes. In this system, viruses would first be divided into *domains* based on the type of their genetic material, similar to the Baltimore system (Baltimore, 1971). Domains would then be divided further into hierarchically distinct divisions, viruses in each division exhibiting little or no evidence for genetic exchange over division boundaries. Within each division, viruses would be assigned to groups, *modi*, that share common sets of features. The unique feature of the proposed system is that viruses could belong to several *modi*, sharing particular characters with other viruses in each *modus*. For example: phage SfV would belong to the domain of dsDNA viruses, division of tailed bacteriophages, and to at least three modi, including I) phages with HK97-like head genes and maturation processes, II) phages with Mu-like contractile tails and III) integrase-mediated temperate phages. Groups of phages that share no *modi* with others could also be placed in their own distinct families. This system is by no means the only possible, but would clearly help in the classification of phages with modular genomes. Alternatively, a single structural gene module, for example the head or tail genes, could be used as the basis of classification, regardless of the relationships of other genes, as proposed by Proux et al. (2002).

1.9.4 Evolutionary analysis based on structural comparisons of viral proteins

Another taxonomical problem is presented by sequences that have originated from a common ancestral gene, but have accumulated mutations so that they are beyond recognition. The amino acid sequence of a given protein may still be recognisable even though the nucleotide sequence has changed beyond recognition, but when the amino acid sequences further diverge from the original sequence, identification of homologous genes by sequence-based methods becomes impossible. Even then, after no sequence similarity is left on the amino acid level, the structure of the protein may still be conserved. It has been proposed that convergent evolution of the exactly same structure, to perform the same task, is extremely improbable and that for this reason, identical structures with dissimilar aa sequences are most probably the products of divergent, rather than convergent evolution (Bannister and Parker, 1985; Lewin, 1985).

Structural comparisons of capsid proteins and architecture have been used in several cases to study the relationships of viruses. The same major capsid protein structure and architectural principle have been found in bacteriophage PRD1 and the eukaryotic-host infecting adenovirus, which also share many other common features, leading to the suggestion that these viruses might share a common ancestor (Benson et al., 1999; Hendrix, 1999; Bamford et al., 2002a; Bamford, 2003). Shortly after this finding, the same capsid protein structure was found in PBCV-1, infecting algae, and sulfolobus Turreted Icosahedral virus (STIV), which is an archaeal virus (Nandhagopal et al., 2002; Khayat et al., 2005). In addition, homology modelling predicted the same structure to be found in several other icosahedral, non-tailed dsDNA viruses of eukaryotic and bacterial viruses (Ravantti et al., 2003; Benson et al., 2004). Thus, this proposed viral lineage encompasses viruses infecting hosts in all three domains of life.

The bacteriophages HK97 and T4 have been shown to have similar capsid protein folds and overall capsid structures, when analysed by X-ray crystallography and cryo-EM (Wikoff et al., 2000; Fokine et al., 2004; Fokine et al., 2005b). Also the folds of bacteriophage phi29 and HK97 capsid proteins have been observed to be similar, except for an additional immunoglobulin-like domain in the phi29 capsid protein (Morais et al., 2005). Analysis of the bacteriophage T5 structure also showed similarity to HK97 and T4 (Effantin et al., 2006). Further, when capsid protein structures from bacteriophages P22, phi29, T4 and HK97 were compared with the herpesvirus capsid protein, a common basic fold was detected, leading to the suggestion that also these viruses could have originated from a common ancestor, predating the division of the Prokarya and Eukarya (Baker et al., 2005).

Naturally, evolution of one sequence is not the same as the evolution of a whole virus, as horizontal transfer of one gene may have occurred between otherwise non-related viruses. However, if several proteins or other features of two viruses show similar or identical evolutionary patterns, this can be regarded as more sound evidence for a common origin of the organisms in question. For example, in addition to the major capsid protein, the genome packaging strategies of herpesviruses and tailed phages are similar, including the use of a portal structure and similarities in terminase sequences, as described above in section 1.4. The proteases of herpesviruses and the tailed bacteriophages have similarly been suggested to be related (Cheng et al., 2004).

Several other viral lineages in addition to the two described above have also been proposed based on structural similarities (Hendrix, 1999; Bamford et al., 2002a). The small ssRNA animal picornaviruses and RNA plant viruses have almost identical coat protein folds, revealing a close evolutionary relationship (Harrison et al., 1978; Abad-Zapatero et al., 1980; Hogle et al., 1985; Rossmann et al., 1985). Similarly, the dsRNA viruses of the animal and plant-infecting *Reoviridae* and the bacteria-infecting *Cystoviridae* share a similar multilayered capsid structure with an inner core exhibiting a unique arrangement of 60 asymmetric dimers of coat protein (Butcher et al., 1997; Grimes et al., 1998; Huiskonen et al., 2006). Two other types of dsRNA viruses with similar structures, the yeast LA-virus of the *Totiviridae*, and the birnaviruses, have also been suggested to be related to the *Cysto-* and *Reoviridae* families and to belong to the same lineage (Bamford et al., 2002a; Naitow et al., 2002; Coulibaly et al., 2005).

The discovery of almost identical structures in viruses infecting evolutionarily very distant hosts has challenged the traditional view that viruses infecting evolutionarily remote organisms are equally distinct from each other. It has been suggested that viruses are ancient organisms, predating the division of cells into Bacteria, Archaea and Eukarya, and that viruses, unlike cellular life, are of polyphyletic origin, i.e. there is no viral LUCA (last universal common ancestor) (Hendrix, 1999; Bamford et al., 2002a; Bamford, 2003).

1.9.5 Evolution of multiple capsid protein types

In cases where more than one type of capsid protein is used, the capsid proteins have often been generated by gene duplication of one original capsid protein gene: for example the structurally very similar three different capsid proteins used by picornaviruses have been proposed to have evolved by duplications and rearrangements of one ancestral gene coding for single a capsid protein type (Hogle et al., 1985; Liljas et al., 2002). In bacteriophage PRD1, several gene duplication and deletion events have been proposed: the two-domain, double β-barrel structure of the P3 major capsid protein has been proposed to be the result of a gene duplication (Benson et al., 1999; Benson et al., 2002), and both the vertex protein P31 and the spike protein P5 have been proposed to have originated from the major capsid protein or its single-domain ancestor (Bamford et al., 1991; Caldentey et al., 2000; Abrescia et al., 2004; Merckel et al., 2005). In bacteriophage T4, it has been shown that such a process can be 'reversed': deletion of the gene for the vertex protein gp24 can be compensated for by mutations in major capsid protein gp23, allowing the originally hexameric gp23 to form both pentamers and hexamers and the pentameric form to be incorporated at the 5-folds instead of the deleted gp24 vertex protein (Fokine et al., 2006). Similarly, the two tail base plate proteins gp10 and gp11, and short tail fiber protein gp12 of T4 have been suggested to have evolved by triplication of a common ancestral protein (Leiman et al., 2006).

1.9.6 Virus-host coevolution

As viruses are totally dependent on their host for survival and replication, virus evolution is naturally tied to host evolution as well. Viruses are in a constant arms-race with their host organisms, the host evolving to evade the virus, and the virus evolving to keep up with the host. Such a co-evolutionary battle can be followed on a reasonably short time scale in the laboratory: continuous cultures of *E. coli* O157:H7 and its specific bacteriophage PP01 have been shown to first lead to the arise of mutant bacteria resistant to the phage and then, after a while, to mutant viruses able to infect the mutant bacteria (Mizoguchi et al., 2003).

As described earlier, the host cells may be an important source of new genes for the virus, either via moron accretion or homologous recombination of similar genes. Indeed, many viruses contain genes more or less recently acquired from their hosts, such as muralytic enzymes and photosynthesis genes (Lindell et al., 2005; Sullivan et al., 2006). In particular, many genes involved in virus-host interactions have very similar cellular homologues (Loessner et al., 1997; Bugert and Darai, 2000). However, viruses also have a significant influence on the evolution of their host organisms. Viruses may control host abundance and population structure (Hennes et al., 1995) by preferentially infecting and killing the dominant genotype, 'killing the winner', or by enabling rare mutations, for example causing phage-resistance (Middelboe et al., 2001) to arise in the population more effectively or by providing an important source of genetic material (Weinbauer and Rassoulzadegan, 2004; Sullivan et al., 2006). Prophages may contribute significantly to inter-strain variation in bacteria, as in the case of *E. coli* O157 strains, where microarray analysis showed that most of the strain-to-strain variation was due to prophages and prophage-like elements (Ogura et al., 2006). Even 8 % of the human genome has been estimated to be of retroviral origin, in addition to other mobile elements found (Lander et al., 2001). Long-term co-evolution of a host-virus pair, where horizontal gene transfer between different viruses is not too extensive, can lead to *co-speciation*, creating similar phylogenetic patterns for the host and virus populations, as seen for example in the case of baculoviruses and the insect hosts they infect (Herniou et al., 2004).

1.10 Bacteriophage PRD1 and the Tectiviridae family

1.10.1 The *Tectiviridae* family

The *Tectiviridae*, the characteristic feature of which is the presence of an internal membrane residing underneath the protein capsid, include viruses infecting both gram-negative and gram-positive bacteria (Bamford, 2005). The type virus of the family is bacteriophage PRD1 (Bamford, 2005). There are five other gram-negative bacteria infecting tectiviruses, PR3, PR4, PR5, L17 and PR772, which are highly similar to PRD1 (Lute et al., 2004; Saren et al., 2005), despite being isolated at different times from distant locations of the world (Olsen et al., 1974; Stanisich, 1974; Wong and Bryan, 1978; Coetzee and Bekker, 1979; Bamford et al., 1981). The tectiviruses infecting gram-positive bacteria are more diverse in sequence (except for Bam35 and pGIL01, which are nearly identical to each other), and include bacteriophage AP50 (Nagy et al., 1976; Bamford, 2005), which infects *Bacillus anthracis*, and the *B. thuringiensis* infecting phages Bam35 (Ackermann et al., 1978; Ravantti et al., 2003), GIL01 (Verheust et al., 2003) and GIL16 (Verheust et al., 2005). A tectivirus infecting *Bacillus acidocaldarius*, phiNS11, has also been reported in the late 1970's (Sakaki et al., 1977), but unfortunately it no longer seems to be available. Recently, new putative members of the *Tectiviridae* family have also been isolated from *Thermus* bacteria in alkaline hot springs (Yu et al., 2006).

1.10.2 Bacteriophage PRD1

1.10.2.1 Introduction to PRD1

Bacteriophage PRD1 is an icosahedrally shaped, non-tailed bacteriophage (Olsen et al., 1974; Bamford, 2005). The genome of PRD1 (accession number AY848689) is a 14927 bp long linear dsDNA molecule with covalently attached 5' terminal proteins and inverted terminal repeats at both termini (Bamford et al., 1983; Bamford and Mindich, 1984; Savilahti and Bamford, 1986; Bamford et al., 1991; Saren et al., 2005). The organisation of the PRD1 genome is shown in Fig. 8a. The genome is replicated by a protein-primed mechanism (Savilahti et al., 1991; Caldentey et al., 1992; Caldentey et al., 1993), similar to what is seen in adenovirus and bacteriophage phi29.

The PRD1 membrane is formed of approximately 50% lipid and 50% phage-specific proteins (Davis et al., 1982; Bamford et al., 1991). The membrane is acquired from the host cytoplasmic membrane, and its phospholipid content may vary according to the host cell type, although the viral membrane is not identical to the host membrane in lipid composition, and certain lipids may be enriched or excluded from the viral internal membrane (Laurinavičius et al., 2004). Most notably, the PG/PE ratio is higher in the phage membrane than in the cell (Laurinavičius et al., 2004). Whether this is due to the higher curvature of the phage membrane leading to exclusion of PE from the outer leaflet, viral modulation of host cell lipid synthesis during infection, or enrichment of PG via preferential binding of viral proteins, as suggested in the case of the related PRD1-type phage PR4, is not clear (Davis and Cronan, 1985; Laurinavičius et al., 2004). During infection, the spherical membrane vesicle undergoes a structural transformation into a tubular structure used as an ejection device and a vehicle for DNA transport into the host cell cytoplasm, and thus represents an alternative DNA delivery method to the tails of the more commonly known *Caudovirales* phages (Lundström et al., 1979; Bamford and Mindich, 1982).

The icosahedral protein capsid of PRD1 is 700 Å in diameter, vertex to vertex, and the 240 copies of major capsid protein P3 are organised in a pseudo T=25 lattice (Butcher et al., 1995; San Martín et al., 2001; Abrescia et al., 2004). P3 has a double β-barrel structure, and three P3 monomers act together to form a pseudo-hexameric trimer (Benson et al., 1999; Benson et al., 2002). The minor capsid protein P30 is an extremely extended protein running along the facet edges: two P30 monomers, each stretching out from one vertex and hooked together at the N/C-terminal ends, form a type of a 'tape-measure', defining the size of the capsid and cementing the facets together (Rydman et al., 2001; Abrescia et al., 2004). The vertices of the virion harbour a spike complex (also called the *vertex complex*) formed of pentameric protein P31, trimeric spike protein P5 and the receptor binding protein P2, a structure equivalent to the pentameric adenovirus penton base and trimeric fiber proteins (Rydman et al., 1999; Bamford and Bamford, 2000; Xu et al., 2000; Sokolova et al., 2001; Merckel et al., 2005). Below the vertex complex, connecting it to the internal membrane and stabilising the vertices resides membrane protein P16 (Abrescia et al., 2004; Jaatinen et al., 2004). Interestingly, P16 is needed only to infect host cells with full-length LPS and cells lacking the O-antigen are susceptible to infection with a P16-deficient mutant, although no difference in virus binding to the cells could be detected (Jaatinen et al., 2004). A schematic representation of the PRD1 virion structure can be seen in Figs. 8b and c.

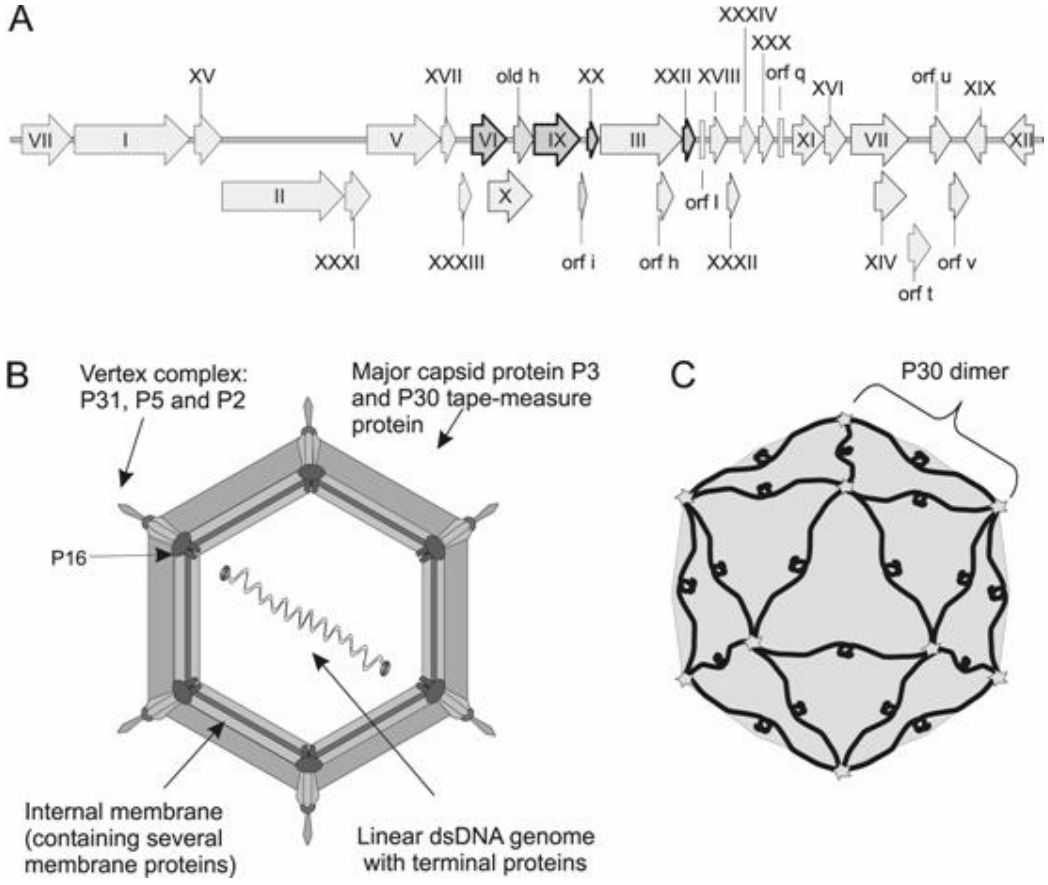

Figure 8. The PRD1 genome and virion structure. A) The organisation of the PRD1 genome. Genes coding for proteins with known or possible packaging-related functions are shown in darker colour. The different levels of the arrows depicting the genes represent the three different reading frames used. B) A schematic representation of the PRD1 virion, and C) organisation of the P30 tape-measure protein in the virion.

PRD1 infects several different species of gram-negative bacteria, including *E. coli* and *S. enterica*, provided that they carry a conjugative plasmid belonging to the IncP, N or W incompatibility groups (Olsen et al., 1974). Plasmid-encoded functions are only required for DNA entry, and the presence of the plasmid is not necessary for the later stages of PRD1 infection (Davis et al., 1982; Lyra et al., 1991). It seems that there is no one single gene responsible for binding PRD1, but as many as 11 plasmid genes belonging to the so-called mating pair formation (Mpf) system may be necessary for PRD1 binding and entry (Grahn et al., 1997). The Mpf proteins of the IncP-type plasmid RP4 have been shown to form a complex connecting the CM to the OM (Grahn et al., 2000), and to be able to increase cell permeability (Daugelavičius et al., 1997), suggesting that the Mpf complex acts as a conductive channel.

The initial, reversible binding of the virion to the cell wall via P2 is followed by irreversible binding, mediated by a still unknown viral protein or mechanism (Grahn et al., 1999; Grahn et al., 2002a). It has been proposed that binding via the common vertex complex (P31, P5, P2, also *spike complex*) to the IncP-encoded DNA transfer complex leads to a conformational change in the vertex structure and removal of the common vertex complex proteins and peripentonal trimers, creating an opening at the vertex that would allow the membrane tail tube to protrude from the capsid (Rydman et al., 1999; Grahn et al., 2002a). Mutants defective in P31 (and thus also lacking P5 and P2) are unable to form the tail-tube and to deliver their DNA efficiently (Rydman et al., 1999). A symmetry mismatch between P2 and P31, making the vertex complex a metastabile structure, has been proposed to be involved in initiating tail tube ejection upon receptor binding (Rydman et al., 1999).

After irreversible binding to the cell, the PRD1 genome must cross three obstructing layers of the cell wall: The OM, the viscous peptidoglycan layer, and the CM. Penetration of the OM has been attributed to P11 (Grahn et al., 2002a), which in turn makes way for P7, a peptidoglycan digesting transglycosylase enzyme (Rydman and Bamford, 2000; Grahn et al., 2002a). When the murein layer has been digested enough to access the CM, the delivery process continues with formation of the membrane tail-tube structure, in which at least proteins P14, P18 and P32 have been shown to be involved (Grahn et al., 2002b; Grahn et al., 2002a).

Protein P15, which is a lytic muramidase responsible for host cell lysis, is part of the virion structure (Mindich et al., 1982a; Caldentey et al., 1994; Rydman and Bamford, 2002). Together with PRD1 holin protein P35, P15 forms a two-component lysis system, similar to what is seen in other dsDNA phages (Rydman and Bamford, 2003; Ziedaite et al., 2005).

The PRD1 virion also contains P9, a putative packaging ATPase, two small membrane proteins, P20 and P22, which have been implicated in DNA packaging or in the stable maintenance of the DNA within the particle, and a 17.7 kDa minor capsid protein P6, the function of which is still unknown (Mindich et al., 1982a; Mindich et al., 1982b). The high-resolution structure of the whole PRD1 virion has been solved by X-ray crystallography, describing also organisation of the lipid membrane (Abrescia et al., 2004; Cockburn et al., 2004).

1.10.2.2 Assembly of the PRD1 procapsid

The first observed soluble precursors for particles are trimers of the major coat protein P3 and the spike protein P5 (Mindich et al., 1982b). Correct folding of PRD1 proteins P3, P5 and P11, as well as the assembly of several small membrane proteins, depends on the host chaperonin proteins GroEL/GroES (Hänninen et al., 1997b). In addition, PRD1 proteins P10, P17, and possibly P33, are involved in capsid assembly but are not part of the assembled procapsid structure (Mindich et

al., 1982b; Caldentey et al., 1999; Bamford et al., 2002b).

In non-membrane containing icosahedral viruses, capsid assembly often invoves scaffolding proteins, which are discarded during or before DNA packaging. In PRD1, capsid assembly is achieved in a very different manner: PRD1 assembly proceeds via formation of virus-specific lipid vesicles covered with non-structural protein P10 and a small amount of capsid protein P3 (5-10% of the final amount) (Rydman et al., 2001). These vesicles have been proposed to be formed from virus-specific membrane rafts in a manner analogous to the formation of clathrin-coated pits (Mindich et al., 1982b; Bamford et al., 1995; Rydman et al., 2001). The rest of the P3 coat proteins together with the minor capsid protein P30 are then added to these 'membrane-scaffolds' displacing P10 from the vesicle in the process (Rydman et al., 2001; Abrescia et al., 2004). As a result, empty procapsids are formed that contain an internal membrane and all other virion components except putative packaging ATPase P9 and the DNA genome with terminal protein P8 covalently attached. A schematic view of the the PRD1 lifecycle is presented in Fig.9.

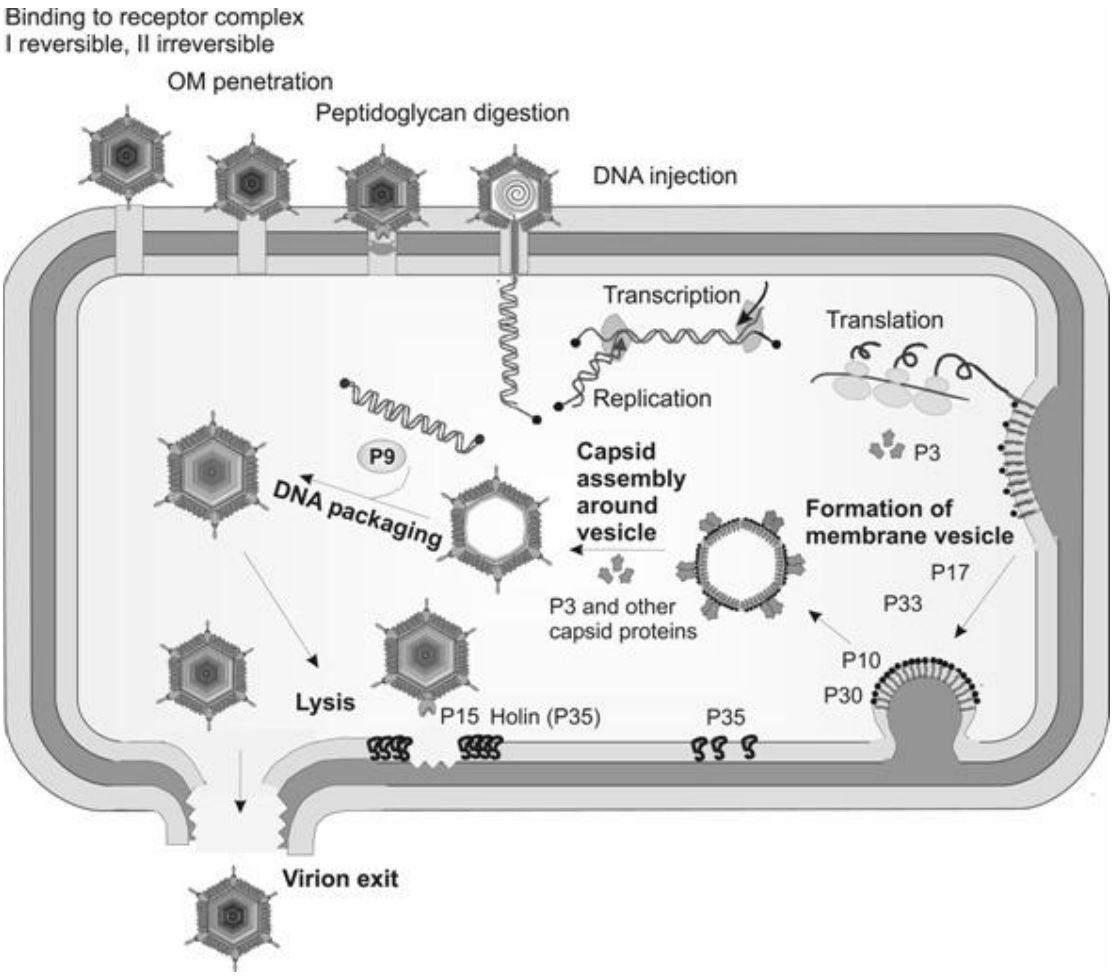

Figure 9. The bacteriophage PRD1 life cycle.

1.10.2.3 PRD1 DNA packaging

Until now, not much has been known about the mechanisms by which PRD1 packages its genome into the icosahedral capsid and the internal membrane vesicle. It has been shown that PRD1 assembly proceeds via empty procapsids, similar to what is seen in other icosahedral dsDNA phages (Mindich et al., 1982b). Three proteins have been identified that may be involved in the packaging protein: a putative packaging ATPase P9, and two small membrane proteins P20 and P22. Lack of P9 leads to formation of particles devoid of DNA, but in the case of P20 and P22 some packaged particles could be seen in sections of infected cells (Mindich et al., 1982b).

Unlike in most other icosahedral dsDNA phages, no capsid expansion or major capsid protein rearrangements are seen in PRD1 in relation to DNA packaging. Instead, the membrane adopts a more angular shape and ordered contacts between the internal membrane and the major capsid protein P3 increase (Butcher et al., 1995). The P3 N-terminus and I1B2 loop areas are the only protein regions undergoing changes upon DNA packaging, as observed by cryo-electron microscopy and image reconstruction of empty and filled particles (San Martín et al., 2001; San Martín et al., 2002). Also, due to the membrane vesicle-mediated assembly mechanism of PRD1, there are no scaffolding proteins present in the procapsid that need to be removed in relation to DNA packaging.

It has previously not been known whether PRD1 has one unique packaging vertex, similar to the situation in tailed phages. Obviously, as PRD1 has no tail, identification of such a structure would be much more difficult. During the course of this thesis work, results obtained by immuno-electron microscopy with gold-conjugated PRD1-specific antibodies were published that showed that proteins P6 and P20 are located at only one vertex of the virion, and thus one of the vertices may differ from the other 11 containing the receptor binding complex (Gowen et al., 2003). The significance of this, together with related results, will be discussed in more detail in the Results and Discussion section.

1.10.3 Evolutionary analysis of the *Tectiviridae-Adenoviridae* lineage

As described above in section 1.9.4, the same double-β-barrel capsid fold seen in bacteriophage PRD1 coat protein P3 has been found in several other viruses, including adenovirus and PBCV-1, which infect eukaryotes, and STIV, which infects an archaeal host (Benson et al., 1999; Nandhagopal et al., 2002; Khayat et al., 2005). These observations have led to the suggestion that these viruses are descendants of a common ancestor and form the so-called *Tectiviridae-Adenoviridae* lineage of viruses. Homology modelling has also revealed that the same protein fold may be found in the coat proteins of viruses such as the gram-positive host infecting tectivirus Bam35 (Ravantti et al., 2003), as well as CIV (belonging to the *Iridoviridae*), african Swine Fever virus 1 (ASFV-1, *Asfarviridae*) and mimivirus (Benson et al., 2004), all viruses of eukaryotes, thus adding potential new members to the lineage. Almost all of these dsDNA viruses have icosahedral capsids and in addition to PRD1, also Bam35, CIV, ASFV-1, mimivirus and STIV have been shown to contain an internal membrane (Ackermann et al., 1978; Carrascosa et al., 1984; Devauchelle et al., 1985; Rohozinski et al., 1989; Van Etten et al., 1991; La Scola et al., 2003; Laurinmäki et al., 2005; Xiao et al., 2005a; Maaty et al., 2006). In addition to probably having similar capsid proteins, the viruses of the *Phycodnaviridae*, *Iridoviridae* and *Asfarviridae* families, and mimivirus, have been shown to share a common core set of genes and have been proposed to be related, and the name 'nucleo-cytoplasmic large DNA viruses' (NCLDVs) has been proposed for them (Iyer et al., 2001; Raoult et al., 2004; Iyer et al., 2006). The same core set of genes can also be found in viruses of the *Poxviridae* family, suggesting that they too should be included in the same NCLDV group (Iyer et al., 2001; Iyer et al., 2006). The NCLDV group was even further expanded by the suggestion that the internal membrane-containing viruses of the *Ascoviridae* family originate from an iridoviral ancestor (Stasiak et al., 2003).

In addition to STIV, another archaeal virus with a structure reminescent of PRD1 has been isolated: the halovirus SH1 has a linear dsDNA genome and virion morphology similar to PRD1 and it contains an internal membrane, although sequence similarity to PRD1, or any other viral proteins is low (Bamford et al., 2005; Porter et al., 2005; Kivelä et al., 2006).

2 AIMS OF THE STUDY

The assembly of bacteriophage PRD1 has been known to proceed via the formation of empty icosahedral particles. However, it has not been known whether PRD1 has a unique portal vertex for DNA packaging, similar to what is seen in tailed phages. As PRD1 does not have a tail, such a unique vertex was not evident from the overall structure of PRD1. Additionally, the receptor-binding structures of PRD1 can be found at all (or at least most) of the twelve capsid vertices, unlike in the case of the tailed phages, where the tail is a receptor binding structure and the single packaging vertex is also the site of DNA ejection from the virion head. The presence of an internal membrane further complicated the picture, as the DNA need not only be inserted into the protein capsid, but it should also be able to traverse the lipid bilayer and be inserted into the internal membrane vesicle within the protein shell. These unique features suggested that the DNA packaging mechanism of PRD1 might differ significantly from those of the tailed phages and herpesviruses, possibly presenting a whole new class of viral packaging systems.

The aim of the study was to find out whether PRD1 uses a unique packaging vertex similar to the tailed bacteriophages, or if some other type of mechanism was used for PRD1 genome encapsidation. Secondly, the objective was to characterise the components and mechanisms involved in the assembly of the packaging machinery, the mechanism of the packaging of the genome into the virus particle and stabilisation of packaged virions. Ultimately, the goal was to set up an *in vitro* DNA packaging system which would allow a more detailed analysis of the PRD1 DNA packaging mechanism.

During the course of this work, we identified a new putative member of the *Tectiviridae* family: pBClin15, which was first published as a linear plasmid as part of the *Bacillus cereus* whole genome sequence (Ivanova et al., 2003), but turned out to be a tectiviral sequence in hiding. The pBClin15 sequence proved later to be an important tool in identifying conserved sequence motifs in the tectiviral ATPases, as other sequences of tectiviruses infecting gram-positive host bacteria were not publicly available at the time. As PRD1 was known to be strictly lytic, the finding of this tectivirus–like plasmid (or putative prophage) in *B. cereus* prompted us to analyse the possibility of lysogeny in Bam35 infection in *B. thuringiensis*, a path not envisioned at the beginning of this work.

3 MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages used in this study are listed in tables 1-3, respectively. Experimental procedures have been described in detail in the original publications and the reader is kindly asked to refer to them. A summary of the methods used is presented in table 4, and the *in vitro* packaging system is briefly described.

Table 1. Bacterial strains used in this study.

Bacterial strain	Description	Relevant properties	Reference	Used in
Salmonella enterica LT2 serovar *typhimurium*				
DS88	SL5676 ΔH2 H1-i::Tn10 (Tc^S) *non rev* (pLM2)	Non-suppressor host for PRD1	Bamford and Bamford, 1990	I, III, IV
PSA(pLM2)	*supE*	Suppressor host for PRD1 mutant *sus621*	Mindich et al., 1976	I, III, IV
DB7154(pLM2)	DB700 *leuA141* (Am) *hisC527* (Am) *supD10*	Suppressor host used for isolation of *sus621*	Winston et al., 1979	I, IV
DB7156(pLM2)	DB700 *leuA141* (Am) *hisC527* (Am) *supF30*	Suppressor host for PRD1 amber mutants	Winston et al., 1979	I
Escherichia coli K12				
DH5α	*supE44* Δ*lacU169* (ϕ80 *lac*ZΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1*	cloning host, with plasmid (pJB15) used for plating packaging reactions	Hanahan, 1983; Sambrook and Russell, 2001	I, III, IV
HMS174	*recA1 hsdR* Rf^R	Cloning host and protein expression	Campbell et al., 1978	I, III, IV
HMS174 (DE3)	*recA1 hsdR* Rf^R	Protein expression	Campbell et al., 1978	IV
HMS17(pcI857) (pTPH19)	*recA1 hsdR* Rf^R (pcI857)	expression strain for PRD1 P19	Pakula et al., 1993	IV
HB101	*supE44 hsdS20* (r_B-m_B) *recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1*	Cloning host	Boyer and Roulland-Dussoix, 1969; Bolivar and Backman, 1979	I, III, IV
Bacillus thuringiensis sv. israelensis				
HER1410		Host for Bam35	Felix d'Herelle Reference Center for Bacterial Viruses, Laval University, Quebec, Canada	II
HER1410_L5		*Bam35 lysogenised in HER1410*		II
HER1410_L7		*Bam35 lysogenised in HER1410*		II
4Q4 (WHO2013-9)		*harbours Bam35-like 15 kbp plasmid and releases virus*	Bacillus Genetic Stock Center, Ohio State University, Columbus)	II

Table 2. Plasmids used in this study.

Plasmid	Description [a)]	Reference	Used in
pLM2	Encodes the PRD1 receptor	Mindich et al., 1976	I, III, IV
pJB15	Encodes the PRD1 receptor	Hänninen et al., 1997a	III, IV
pSU18	cloning vector, p15A replicon, Plac, Cm^R	Bartolomé et al., 1991	I, III, IV
pJJ2	expression vector, Ap^R,	Ojala et al., 1993	IV
pMS470□8	construction platform for plasmid pMG60	Balzer et al., 1992	III
pMG60	expression vector, ColE1 replicon, Ptac, Ap^R, T7 RBS	III	III
pMG119	PRD1 gene *XX* (nt 8445-8588) in pSU18	obtained from M. Grahn, I	I
pMV11	PRD1 gene *XXII* (nt 9784-9944) in pSU18	I	I
(pcI857)	Encodes thermo-sensitive repressor for heat induced expression of proteins under the λ P_L promoter	Remaut et al., 1983	IV
pPLH101	expression vector with λ P_L promoter,	Liljeström et al., 1988	IV
pTPH19	PRD1 gene *XIX* cloned into pPLH101	Pakula et al., 1993	IV
pNS1	PRD1 gene *IX* including preceding SDS sequence (nt 7624-8320) cloned into pSU18	I	I, III
pNS21	PRD1 gene *VI* including preceding SD sequence (nt 6769-7284) cloned into pSU18,	IV	IV
pNS22	PRD1 gene *VI* (nt 6784-7284) cloned into pSU18	IV	IV
pNS62	PRD1 gene *VI* (nt 6784-7284) cloned into pJJ2	IV	IV
pNS96	PRD1 gene IX (nt7638-8320) cloned into pMG60	III	III, IV

P9 mutant plasmids	Description	Mutation		
pNS901	as complementation plasmid pNS1, but with altered P9	(K22R)	III	III
pNS902	"	(K22A)	III	III
pNS903	"	(Y74A)	III	III
pNS904	"	(D103A)	III	III
pNS905	"	(E104Q)	III	III
pNS906	"	(T120A)	III	III
pNS907	"	(R123A)	III	III
pNS908	"	(R123K)	III	III
pNS909	"	(Q134A)	III	III
pNS910	"	(R135K)	III	III
pNS911	"	(R135A)	III	III
pNS912	"	(P208R)	III	III
pNS913	"	(K17R)	III	III
pNS914	"	(K17A)	III	III
pNS915	"	(K17Q)	III	III
pNS916	"	(Y72A)	III	III
pNS917	"	(K125A)	III	III
pNS918	"	(Q134E)	III	III
pNS919	"	(F149V)	III	III
pNS920	"	(F150A)	III	III
pNS921	"	(F153A)	III	III
pNS922	"	(Y74F)	III	III
pNS923	"	(D103E)	III	III
pNS924	"	(E104D)	III	III
pNS926	"	(3 aa C-term. deletion)	III	III
pNS927	"	(7 aa C-term. deletion)	III	III
pNS933	as expression vector pNS96, but with altered P9	(R135K)	III	III
pNS936	"	(P208R)	III	III
pNS942	"	(K17Q)	III	III
pNS945	"	(Y74F)	III	III
pNS948	"	(K125A)	III	III

a) Numbers refer to the PRD1 genome coordinates (**AY848689**)

Table 3. Bacteriophages used in this study.

Bacteriophage	Description	Reference	Used in
PRD1			
wt	wild type	Olsen et al., 1974	I, III, IV
PRD1-1	PRD1 with lacz-α insertion (nt 6309), (used for production of DNA for packaging reactions)	Bamford and Bamford, 2000	III, IV
sus1	amber mutation in gene *IX*	Mindich et al., 1982a	I, III, IV
sus42	amber mutation in gene *XXII*	Mindich et al., 1982a	I
sus148	amber mutation in gene *XVIII*	Mindich et al., 1982a	I
sus234	amber mutation in gene *VII*/*XIV*	Mindich et al., 1982a	I
sus400	amber mutation in gene *XX*	I	I
sus471	amber mutation in gene *II*, opal mutation in gene *VII* ($P2^-/P7^-$ when propagated in non-suppressor host, $P7^-$ in suppressor host)	Rydman and Bamford, 2000	I
sus525	amber mutation in gene *XXXI*	Rydman et al., 1999	I
sus526	amber mutation in gene *XX*	I	I
sus539	amber mutation in gene *II*	Grahn et al., 1999	I
sus607	amber mutation in gene *XI*	San Martín et al., 2002	I
sus621	amber mutation in gene VI, two missense mutations in gene VI	IV	IV
sus690	amber mutation in gene *V*	Bamford and Bamford, 2000	I
[*lac*Zα]-9	amber mutation in gene *XXXII*	Grahn et al., 2002b	I
Bam35c	wt Bam35c	Ravantti et al., 2003	II

Table 4. Summary of methods used in this study

Method	Used in
Growth and purification of PRD1	I, III, IV
NTG mutagenesis and isolation of PRD1 mutants	I, IV
in vivo complementation analysis of PRD1 mutants	I, III, IV
PCR and other standard molecular cloning techniques	I, II, III, IV
DNA sequencing	I, III, IV
Agarose gel electrophoresis and EtBr staining	I, II, III, IV
Purification of total genomic DNA from *Bacillus thuringiensis* strains	II
Purification of PRD1 DNA by phenol-ether extraction and ethanol precipitation	III, IV
Quick Change® site-directed mutagenesis (Stratagene)	III
Recombinant protein expression	III, IV
SDS-PAGE and Coomassie Brilliant Blue staining	I, III, IV
Western blotting	I, III, IV
N-terminal amino acid sequencing	IV
Protein concentration measurement with the Bradford assay	I, III, IV
Transmission electron microscopy	I, IV
Polyclonal antiserum production	I, IV
Purification of recombinant P6	IV
In vitro DNA packaging	III, IV
Bam35 production	II

The PRD1 *in vitro* packaging system

The *in vitro* packaging system developed for PRD1 consists of empty procapsids from a packaging ATPase P9 deficient mutant (*sus1*) infection (cell extract), recombinant packaging ATPase P9 extract and purified PRD1 mutant DNA containing a *LacZα*-insert. The reaction was buffered with Tris-HCl, pH 7.2, and salts ($MgCl_2$, $CaCl_2$ and NaCl) and PEG were added to the reaction, as described in (III). Energy was provided in the form of ATP, and +37° C was used as the standard incubation temperature. Plating on a host providing the LacZω-fragment (*E. coli* DH5α (pJB15)), which complemented the LacZα–fragment from the virus, was used to distinguish between *in vitro* packaged virions and any background virions from the particle extract, as due to the absence of the *LacZα*-insert the latter would produce only white plaques. As plating efficiency on *E. coli* DH5α (pJB15) was significantly lower than for the standard *Salmonella* PRD1 host, reactions were plated on both hosts to analyse maximum packaging capacity. A schematic picture of the developed *in vitro* packaging system is shown in Fig.10.

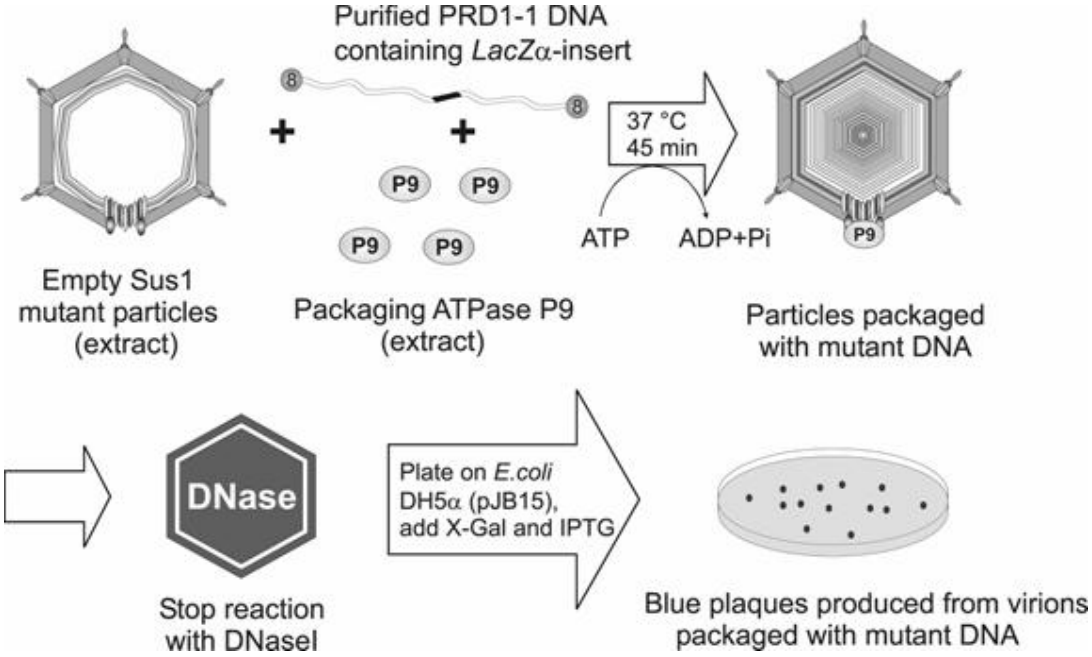

Figure 10. PRD1 *in vitro* packaging.

4 RESULTS AND DISCUSSION

4.1 Isolation and characterisation of P20$^-$ mutants defective in DNA packaging (I)

The small membrane proteins P20 and P22 have been suggested to play a role in DNA packaging. Typically, the functions of different PRD1 proteins has been analysed by creating suppressor-sensitive (amber) mutants, essentially conditional-lethal mutants, which allow analysis of the virus life cycle in the absence of the protein of interest in non-suppressor host cells. Two suppressor-sensitive mutants in gene *XX*, *sus30* and *sus78*, have been reported earlier (Mindich et al., 1982a). However, when the nt sequence of the packaging-related genes in these mutants was determined, it was found that they did not contain any mutations in gene *XX* (data not shown). It was thus necessary to obtain new mutants, which could be used to analyse the role of P20. NTG mutagenesis was performed on PRD1 and two new amber mutants in gene *XX* were isolated, as described in (I), and subsequently named *sus400* and *sus526*.

When the new P20$^-$ mutant particles were further analysed, it was shown that in addition to the absence of P20, they lacked both minor capsid protein P6 and putative packaging ATPase P9. However, infected cells were found to express P6 and P9 at wt amounts, clearly showing that the absence of P6 and P9 in the P20$^-$ mutant particles was due to the inability to assemble these proteins onto the particles, not a defect in protein expression. The P20$^-$ mutants were found to be defective in DNA packaging, producing only empty viral particles, as seen in both thin-sections of infected cells and purified particles.

4.2 Identification and characterisation of a unique packaging vertex (I)

The new P20$^-$ mutants and all other available mutants in PRD1 structural proteins were analysed by SDS-PAGE and Western blotting with all available antibodies against PRD1 structural proteins.

Mutants shown to be defective in DNA packaging (P9$^-$, P20$^-$ and P22$^-$) were found to contain a wt amount of the common vertex complex proteins P31, P5 and P2. The packaging ATPase P9 deficient mutant was not found to lack any other structural proteins, whereas the P20$^-$ and P22$^-$ mutants were found to lack both minor capsid protein P6 and putative packaging ATPase P9, but not to have deficiencies in any other structural proteins, including the common vertex complex proteins. Correspondingly, the vertex complex protein mutants (P31$^-$, P5$^-$ and P2$^-$) were shown to have wt amounts of P22, P9 and P6. In both the packaging protein mutants and vertex complex mutants, no other structural proteins were affected. The lack of P6 in packaging-deficient P20$^-$ and P22$^-$ mutant particles suggested that it too was part of the complex responsible for DNA packaging.

In wt PRD1 infection, a small amount of empty particles (10-20% of all particles produced) is always seen (Mindich et al., 1982b). If lysis is delayed, these particles proceed to be filled with DNA. When DNA-filled and empty wt particles were compared, the only difference in protein composition in addition to the absence of the genome-terminal protein, was found to be the absence of putative packaging

ATPase P9 from the empty particles, and no other structural proteins were missing. Similarly, in the P9 deficient mutant, the only protein missing from the particles was P9, suggesting that P9 is the last protein added to the virion during the transition from empty particles to DNA-filled virions.

Concurrent with this work, the PRD1 virion was analysed by immunogold-labelling and cryo-EM (Gowen et al., 2003). Gowen et al. (2003) showed for the first time that one vertex of the PRD1 virion was different from the others: proteins P6 and P20 were found to be located at only one vertex, whereas the receptor-binding protein P2 could be seen to be located at multiple vertices. As our analysis of PRD1 mutants showed that lack of proteins P20 or P22 led to loss of proteins P6 and P9, but not loss of the common vertex complex proteins (P31, P5 and P2), and *vice versa*, it was concluded that P22 and P9 were also located at the same unique vertex of the virion. A unique packaging vertex, containing the packaging ATPase P9 and the minor capsid protein P6, and extending to the internal membrane via the small membrane protein P20 and P22, was thus proposed for PRD1.

As the absence of any of the analysed packaging-related proteins (P20, P22, P6, P9) had no effect on the common vertex complex proteins (P31, P5 and P2), and *vice versa*, it is suggested that the packaging proteins are most probably located at one separate vertex, while the common vertex complex proteins occupy the other eleven vertices, instead of one of the twelve vertices carrying both the vertex complex proteins and the packaging machinery. The sheer size of the vertex complex and the steric hindrances involved in fitting both structures at the same vertex also supports this model. An updated view of the PRD1 virion structure, including now the unique packaging vertex, is presented in Fig.11.

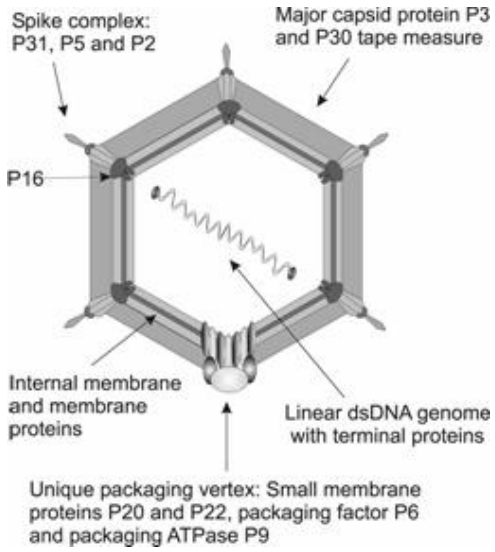

Figure 11. Schematic representation of the structure of PRD1 with the unique vertex. The exact arrangement of the packaging vertex proteins is not known, and this picture is only a schematic representation of the overall structure.

The relationship between the small membrane proteins P20 and P22 remains somewhat obscure, as the antibodies for P20 that were used in immuno-gold labelling (Gowen et al., 2003), were not functional in Western blotting. Both P20 and P22 were clearly found to be necessary for the incorporation of P6 and P9 into the capsid. However, it was shown that P22 binding was not dependent on the presence of P20, as $P20^-$ mutants contained wt amounts of P22. Unfortunately, the lack of P20-specific antibodies working in Western Blotting prevented the inquiery into whether $P22^-$ particles correspondingly contained P20, or not. The presence or absence of P20 and P22 cannot be reliably determined from regular SDS-PAGE gels either, but perhaps in the future, for example 2D SDS-PAGE could be used to clarify the question.

4.2 Identification of plasmid pBClin15, a new member of the Tectiviridae family (II)

When database searches were performed with the Bam35 and PRD1 putative ATPase sequences as seed, an ORF in the 15 kbp linear plasmid pBClin15 of *B. cereus*, which had been published in conjunction with the *B. cereus* whole genome sequence (Ivanova et al., 2003), was retrieved. When we analysed the pBClin15 sequence more carefully, this plasmid was found to contain multiple other ORFs with similarity to Bam35 genes (Ravantti et al., 2003), and essentially in the same order, making it remarkably similar to Bam35 (II).

We re-annotated the pBClin15 sequence with comparisons to the Bam35c sequence as a basis, and identified several ORFs that were not annotated as ORFs in the original pBClin15 sequence, but matched existing Bam35 genes. When the pBClin15 ORFs and Bam35 genes were compared on the amino acid level, the sequences were found to have 45-81 % identity, the most conserved sequences being those assigned to capsid proteins and DNA packaging functions in Bam35, in addition to a LexA-repressor-type sequence, which was the most conserved sequence between Bam35 and pBClin15. The comparison of Bam35 genes and pBClin15 ORFs is shown in Fig.12.

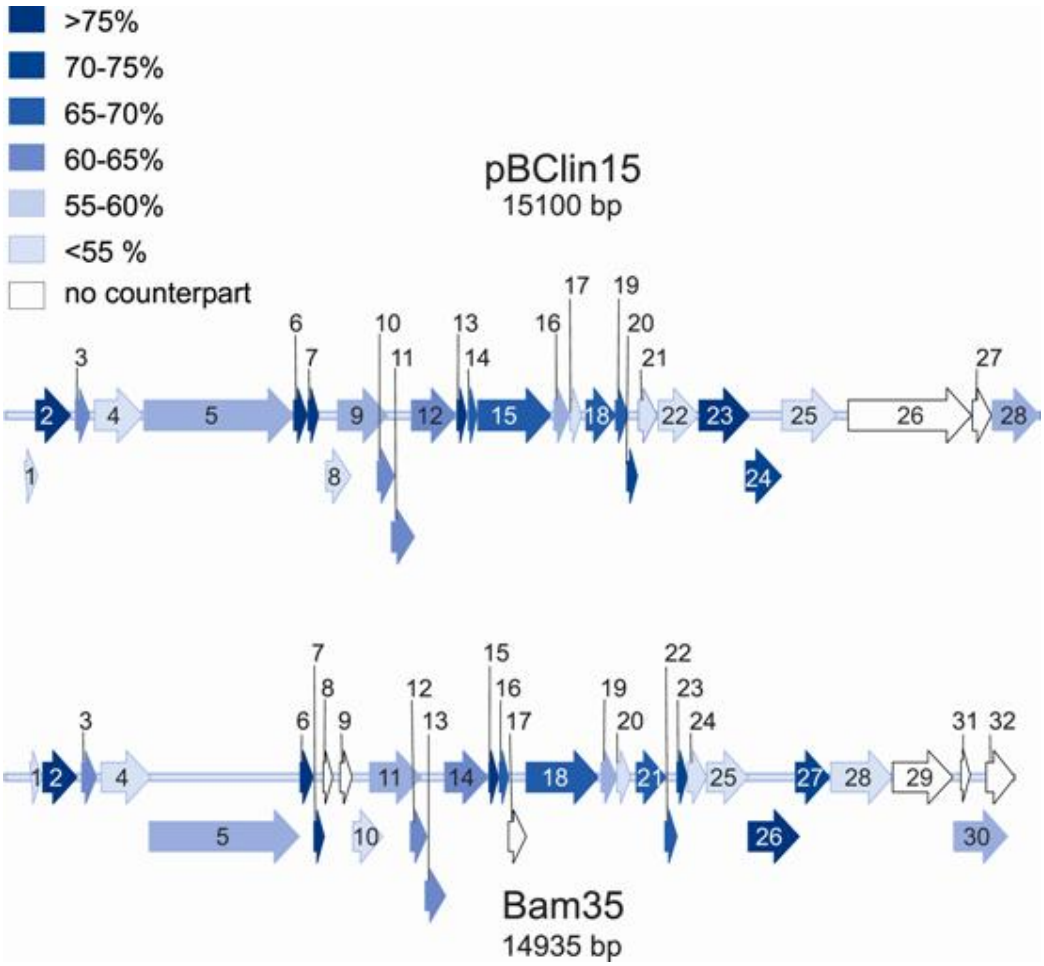

Figure 12. Comparison of the pBClin15 ORFs and Bam35c genes. Redrawn from (II).

Homology modelling of the pBClin15 putative coat protein sequence, based on an earlier model of the Bam35 coat (Ravantti et al., 2003), showed that the pBClin15 putative coat protein most probably had the same double β-barrel fold as the PRD1 and adenovirus capsid proteins (II).

The pBClin15 plasmid did not contain inverted repeats at the ends of the linear molecule, as Bam35 and PRD1 do. In PRD1, the ITRs have been shown to contain the minimal origin of replication (Yoo and Ito, 1991). It has also been suggested that during replication, the PRD1 ITRs could base-pair to form a pan-handle structure (Savilahti and Bamford, 1993). The lack of ITRs in the pBClin15 sequence suggests that pBClin15 may possibly be a defective phage, and not a functional prophage. Clearly, the pBClin15 sequence was still easily identifiable as Bam35-like, and did not contain any other major deletions or deletorious rearrangements as compared to the Bam35 genome, so any inactivation of the phage should have been evolutionarily rather recent. Alternatively, pBClin15 could still be inducible, but it remains to be shown whether it can actually produce infectious viral particles. Prophage or defective phage, pBClin15 proved to be a valuable addition to the tectiviral family of sequences and a helpful tool in subsequent bioinformatical analysis of the PRD1 P9 type ATPases (III).

4.3 Bam35 is able to exist in a prophage state (II)

It had already earlier been proposed that the tectivirus Bam35, which infects gram-positive hosts, might be able to lysogenise (Ackermann et al., 1978), and similarly to the pBClin15 sequence, the Bam35c genome also contained a gene coding for a LexA-type repressor type protein (Ravantti et al., 2003). The finding of a Bam35-like plasmid prompted us to explore the possibility of Bam35 lysogenisation in more detail.

It was shown that Bam35c could enter a lysogenic state in *B. thuringiensis*. The Bam35c DNA genome did not integrate into the host chromosome but was maintained as a stable linear plasmid. After liquid culture for over eight hours, the lysogenised strains started releasing viruses into the supernatant. Similarly, when other *B. thuringiensis* strains obtained from the Bacillus Genetic Stock Centre (Ohio State University, Columbus) were analysed, four of the seven strains tested were found to release virus. One of these virus-releasing strains, 4Q4, was found to contain a plasmid of approximately 15 kbp, similar to the length of Bam35 and PRD1 genomes. When analysed by PCR with Bam35-specific primers, strain 4Q4 was found to contain Bam35-like sequences. Unfortunately, the nucleotide sequence of 4Q4 is not available, so no detailed comparisons to pBClin15 or Bam35 could be made.

The finding that Bam35 could lysogenise, and that Bam35-type prophages were present in other *Bacillus* strains, raised the questions as to how the Bam35 carrier state was maintained. The Bam35 genome most probably contains terminal proteins (Ravantti et al., 2003), analogous to the covalently linked 5' terminal proteins in PRD1 that are used for protein-primed DNA replication, similar to phi29, adenovirus and certain linear plasmids (Salas, 1991). The possibility of the protein-primed mechanism working also in the Bam35 lysogenic state was intriguing, as this mechanism had not so far not been reported for prophages (Casjens, 2003). Subsequent work with Bam35 has shown that although Bam35 can enter lysogenic strains, late functions of the phage, such as lysis, are repressed in lysogenic cells upon superinfection, as compared to Bam35 infection of the corresponding non-lysogenic host cells (Gaidelyte et al., 2005).

As described previously, Bam35 and the other gram-positive bacteria infecting tectiviruses, which all seem to be lysogenic, display more sequence variation than the tectiviruses of gram-negative hosts, which are strictly lytic. This is somewhat reminescent of the

situation in other groups of bacteriophages where the genomes of temperate phages exhibit more mosaicism than those of viruses with a lytic life-style (see section 1.9.1). However, as only a relatively small group of tectiviruses has so far been studied, this picture may not be the final one. Alternatively, the situation may also be due to the gram-positive hosts infecting tectiviruses being much more ancient than their relatives infecting gram-negative hosts.

4.4 Development of an in vitro packaging system for PRD1 (III)

An *in vitro* packaging system for PRD1 was developed that consisted of a cell extract from a packaging ATPase P9 deficient mutant infection containing empty particles, recombinant packaging ATPase P9 extract, and purified PRD1 mutant DNA containing a *LacZα*-insert (see Materials and methods, Fig. 10). This is the first *in vitro* packaging system developed for an internal-membrane containing bacteriophage.

The energy for PRD1 DNA translocation into the capsid was found to be specifically provided by adenine nucleoside triphosphates and no signal could be obtained with the three other NTPs tested (GTP, CTP and UTP). The reaction was less stringent with respect to the sugar moiety, as both dATP and ddATP supported the packaging reaction, albeit with a somewhat lower efficiency than ATP. The reaction was found to be insensitive to the addition of RNases (RNaseA, RNaseI or RNaseIII) suggesting that there are no RNA molecules involved in PRD1 packaging, as there is in bacteriophage phi29 (Guo et al., 1987b).

The addition of Mg^{2+} ions, but not Ca^{2+}, to the reaction was found to be important, as was as the total salt concentration of the reaction. Polyethylene glycol enhanced the packaging efficiency significantly, similar to, for example, the T7 *in vitro* packaging system (Shibata et al., 1987), and buffering of the reaction was found to be necessary, although a rather broad range of pH (6-8) was tolerated without significant loss in packaging efficiency (III).

The PRD1 *in vitro* packaging assay was found to be comparable in efficiency to *in vitro* systems published for other bacteriophage systems. Titers up to 2.4×10^8 pfu/ml of packaging reaction or up to 4.8×10^7 pfu/µg DNA were achieved, whereas titers of 1×10^7 and 6×10^7 pfu/ml of packaging reaction have been reported for the SPP1 and phi29 *in vitro* packaging systems, respectively (Lee and Guo, 1995a; Dröge and Tavares, 2000), and commercial packaging kits available for bacteriophage lambda advertise titers ranging from 10^8 (Packagene™ Lambda DNA packaging system, Promega) to 10^9 pfu/µg DNA (MaxPlax™ Lambda packaging extracts kit, Epicentre). Despite the rather high titers obtained, it was calculated that only up to 0.08% of genomes and 0.04% of the procapsids were productively packaged, yielding infective virions. The limiting factor in the reaction was the amount of PRD1 DNA, and a saturating concentration could not be reached. This was probably due to damage to or loss of the genome terminal protein P8 during DNA isolation and purification, as it had previously been observed that P8 is highly adhesive and DNA-isolation with P8 attached is very challenging (unpublished observations). It is yet unclear whether P8 plays a direct role in DNA packaging, but this could not be ascertained by, for example, attempting to package protease-treated DNA, as the current *in vitro* system relies on plaque formation for detection, and P8 is strictly required for genome replication. Whether any of the non-structural viral proteins are involved in the PRD1 packaging process could not yet be analysed, as the *in vitro* packaging system developed used extracts from an ATPase deficient mutant infection to produce procapsids, and non-structural PRD1 proteins were thus present in the system.

4.5 Evolutionary analysis of putative packaging ATPases in the Tectiviridae-Adenoviridae lineage (III)

4.5.1 Bioinformatical and mutational analysis of PRD1 P9 (III)

The amino acid sequence of PRD1 P9 was aligned with the corresponding putative ATPase sequences of Bam35 and pBClin15, the PRD1-related phages/ plasmids replicating in gram-positive hosts (Fig. 13). Three regions comprising several similar or identical residues were observed: two of the conserved regions could easily be identified as the classical Walker A (<hydrophobic stretch> [GA]XXGXGK[ST]) and Walker B (<hydrophobic stretch>D[ED]) ATPase motifs (Walker et al., 1982; Gorbalenya and Koonin, 1989). The third region of similarity found (starting ALXTGRSK and ending DR), located downstream of the Walker B motif, could not be identified as any previously described ATPase motif, and was assigned as the P9-specific motif. Several other short regions of identical amino acids (e.g. IY-PI) preceding the Walker B motif were detected.

These motifs were used as targets for mutational analysis and single amino acid changes were introduced to the conserved residues of the three motifs in PRD1 P9. The resulting mutant constructions were tested by an *in vivo* complementation assay using a P9-deficient PRD1 mutant and a subset of the mutants proteins was further tested in the *in vitro* packaging system. The conserved regions of PRD1 P9 identified by sequence analysis were shown to be essential and only few amino acid changes were allowed within these motifs, as described in more detail in (III). Essentially, it was found that the most conserved residues within the motifs tolerated the least changes, with even rather conservative aa changes leading to loss of P9 activity in the *in vivo* complementation and *in vitro* packaging assays.

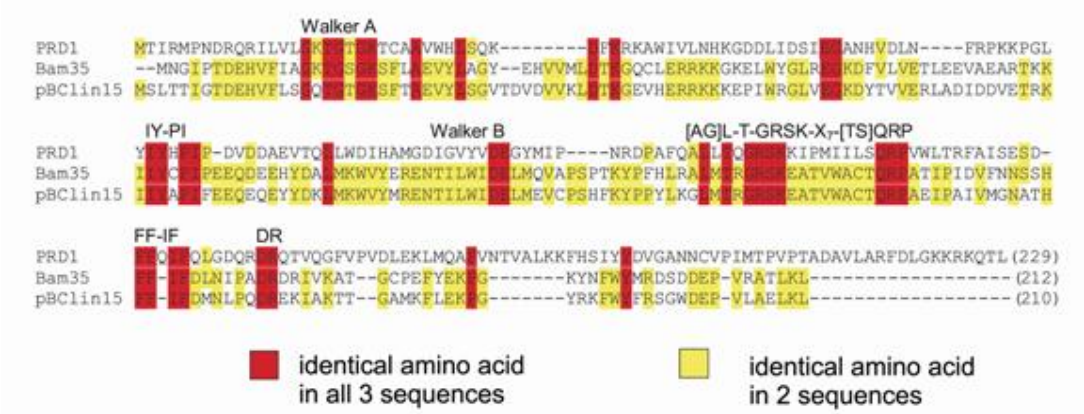

Figure 13. Comparison of tectiviral ATPase sequences. Redrawn from (III).

4.5.2 Database searches for P9-like proteins (III)

Iterative database searches with PSI-Blast (Altschul et al., 1997) using the PRD1 ATPase P9 amino acid sequence as the seed were performed (III). In the first PSI-Blast search, only other tectiviral ATPase sequences were retrieved, but in the next iterative search round several sequences from viruses of the *Ranavirus* genus (belonging to the *Iridoviridae* family) were identified. In the third iteration, phycodnaviral, ascoviral and other iridoviral sequences could be retrieved, in

addition to a putative ATPase sequence from mimivirus, followed by poxvirus sequences. The B164 protein of archaeal virus STIV and the P9 protein of the membrane-containing dsDNA *Corticovirus* PM2 were also retrieved. A similar pattern of search results could be obtained by starting the database search with the Bam35 putative ATPase sequence. The genome sequence of archaeal virus SH1 was at the time not completed and deposited in the database, but the putative ATPase sequence of SH1 was also used as starting point for database searches, and the first viral sequence retrieved was PRD1 P9. It is of specific interest that almost all the sequences retrieved with the tectiviral sequences as seed belonged to icosahedral dsDNA viruses with internal membranes, essentially the same group of viruses which had earlier been linked to PRD1 via the similar double β-barrel coat protein fold, as described in sections 1.9.4 and 1.10.3. With non-stringent search parameters, the adenovirus IVa2 protein could also be retrieved. However, due to an additional N-terminal sequence, IVa2 differed significantly in length from the other viral sequences obtained.

No packaging ATPases of the tailed phages (such as phi29, lambda, P22 or T4) or eukaryotic viruses belonging to other than the above-mentioned virus families (such as the *Herpesviridae*) could be retrieved with the PRD1 or Bam35 sequences with any of the search parameters used. And *vice versa*, the PRD1 putative ATPase could not be retrieved from the databases using phi29, T4 or herpesvirus packaging ATPase/terminase sequences, further supporting the suggestion of the viruses belonging to separate evolutionary lineages.

4.5.3 Identification of a packaging ATPase motif in dsDNA membrane viruses of Bacteria, Eukarya and Archaea (III)

The tectiviral ATPase sequences were aligned with representative sequences of the above described putative viral ATPase sequences retrieved with PSI-Blast. Additionally, putative ATPase sequences from other eukaryotic and archaeal viruses that have been suggested to be structurally related to PRD1 or to the eukaryotic viruses retrieved in the database search described above, such as ASFV and the halovirus SH1, were included in the alignment. For the comparison, either the type virus of each family or genus was chosen, or in some cases, the member of the family first picked up in the iterative database search with PRD1 P9. The comparison of putative dsDNA membrane virus ATPases is presented in Fig.14.

Walker A and B motifs could be detected in all compared sequences at similar positions to the motifs identified in the tectiviral sequences (Fig.14). The P9-specific motif identified in the tectiviruses is similar in sequence and position to the A32 motif identified earlier in the VV A32 protein and putative ATPases of certain other eukaryotic (NCLDV) viruses (Koonin et al., 1993; Iyer et al., 2001), and all the analysed sequences contained a similar motif. The function of these putative ATPases in eukaryotic viruses has not yet been experimentally verified, except in the poxviruses, where the VV A32 protein has been shown to play a role in genome encapsidation (Cassetti et al., 1998). A similar motif was also found in the archaeal virus sequences of STIV protein B164 and the putative SH1 ATPase. This third motif could not been found in the adenovirus IVa2 sequence, and for this reason, IVa2 was not included in the final alignment. As all the viruses having putative ATPase sequences carrying the third motif (the P9/A32-specific motif) contained an internal membrane, we suggest that this region is a membrane-virus specific packaging ATPase motif.

In all the compared ATPases, the packaging ATPase motif (P9/A32) is

composed of an arginine residue, usually preceded by a glycine, followed by a strictly conserved glutamine 11 amino acids downstream, and a conserved phenylalanine 17-19 amino acids further downstream (see Figure 14.). During the preparation of this thesis, a sequence analysis study was published that grouped the above mentioned (putative) viral ATPases as a clade of a new FtsK-HerA superfamily of ATPases (Iyer et al., 2004). The conserved glutamine and the preceding highly conserved arginine were proposed to be a distinguishing factor for the whole superfamily when compared to other P-loop ATPases (Iyer et al., 2004). The arginine residue was suggested to be part of a so-called arginine finger found in the AAA+ ATPase superfamily proteins, and the conserved glutamine to be structurally equivalent to the polar residues possibly playing a role in sensing the triphosphate moiety of the bound nucleotide and triggering ATP hydrolysis in the AAA+ ATPases SFI and SFII helicases (Iyer et al., 2004).

In their analysis, Iyer and co-workers (2004) positioned the putative dsDNA membrane virus ATPases in their own separate clade, with their closest relatives being the clade of FtsK/SpoIIIE-type cellular proteins. Interestingly, but maybe not so surprisingly, the FtsK/SpoIIIE proteins are involved in transport of DNA across membranes or through septa, during cell division and sporulation, respectively (Begg et al., 1995; Wu et al., 1995), and they have been suggested to function as multimeric (probably hexameric) DNA pumps (Bath et al., 2000; Aussel et al., 2002).

Despite the many structural similarities between PRD1 and adenovirus, and some degree of sequence similarity between specific proteins of PRD1 and adenovirus, we were not able to find a P9/A32 type packaging motif in the adenovirus IVa2 protein. When analysing the relationships of the FtsK/HerA superfamily of ATPases with other types of ATPases, Iyer et al. (2004) place adenovirus IVa2 in a separate class, distinct from the Ftsk/HerA superfamily containing the putative ATPases the membrane-containing dsDNA viruses, but also separate from the terminases of tailed phages, similar to our analysis.

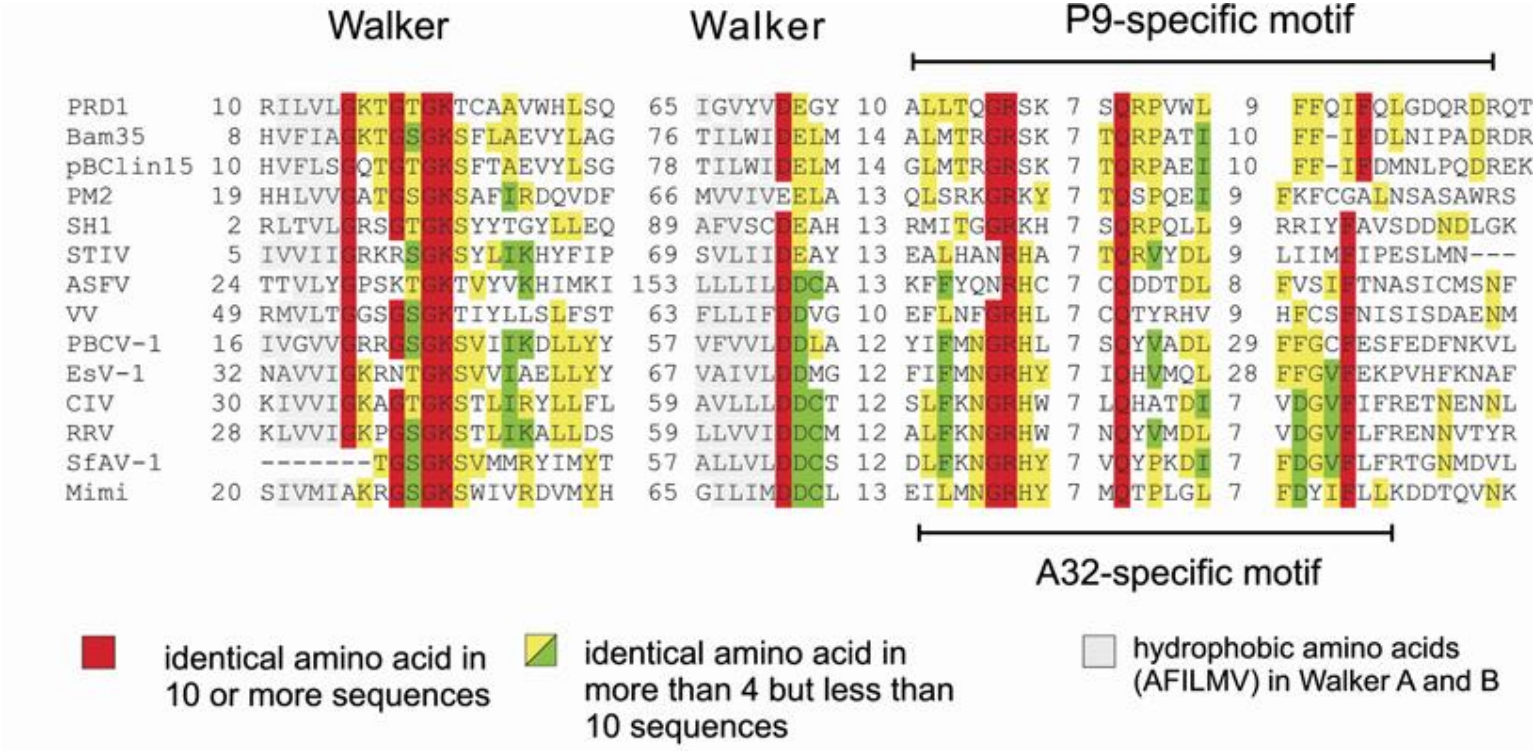

Figure 14. Alignment of P9-type putative ATPase sequences from membrane-containing dsDNA viruses of bacterial, archaeal and eukaryotic origin. Amino acid residues identical in 10 or more of the sequences are depicted in red, and residues identical in more than 3 but less than 10 sequences are depicted in yellow and green (which is used in the case of two groups of more than 3-9 identical amino acids).
Putative ATPases and their accession numbers: PRD1 P9 (P27381), Bam35c orf 14 (NP_943760), pBClin15 orf12 (NP_829897), SH1= archeal virus SH1 orf 17 (YP_271874), STIV= Sulfolobus Turreted Icosahedral Virus B164 (AAS89100), ASFV= African Swine Fever Virus pB354L (NP_042772), PBCV-1= Paramecium Bursaria Chlorella Virus 1 A392R (NP_048749), ESV-1= Ectocarpus siliculosis Virus 1 orf 26 (NP_077511), CIV= Chilo Iridescent Virus ATPase 075L (T03048), SfAV-1= Spodoptera frugiperda Ascovirus 1 ATPase 3 (CAC84470), RRV= Regina Ranavirus ATPase (YP_003858), VV= Vaccinia Virus A32 (P21055), Mimi= Acantamoeba polyphaga mimivirus putative ATPase (AAV50705), VV=Vaccinia virus A32 virion packaging ATPase (AAV50705). Redrawn with corrections from (III).

The similarities between the putative packaging ATPases of the membrane-containing eukaryotic, archaeal and bacterial viruses suggest a similar mechanism for the packaging of their dsDNA genomes. The fact that these viruses have an internal membrane, and that packaging enzymes of tailed phages and herpesviruses could not be retrieved in the database search with PRD1 P9 or other tectiviral ATPases suggests that the DNA packaging enzymes of these viruses represent a separate group with possibly a very different packaging mechanism from the much-studied tailed phage/herpes group of packaging systems.

Further, the presence of a similar ATPase sequence in internal membrane viruses infecting hosts in all domains of life suggests that these viruses may have had a common ancestor carrying this feature that existed before the separation of the current domains of life. Alone, the presence of similar ATPase sequences would probably not be so significant, but as a similar evolutionary relationship for these viruses has been suggested based on their similar coat protein folds and capsid architectures, together these findings provide strong support for common ancestry. Even the poxviruses with their amorphous structures, which at first glance seem to differ drastically from the icosahedral viruses, have actually been shown to go through spherical assembly intermediates covered in an ordered honeycomb protein lattice (Heuser, 2005; Szajner et al., 2005). The only exception is provided by adenovirus, which has a coat protein fold and virion architecture that are similar to those of the other members of this virus lineage, but lacks a membrane. Compared to the putative ATPases of the *Tectiviridae-Adenoviridae* lineage, the adenovirus IVa2 putative ATPase sequence is more remote, although probably not totally unrelated. Therefore, it is possible that the ancestral adenovirus may originally have contained a membrane, which was subsequently lost, possibly due to changes in the entry mechanism of the ancestral adenovirus that rendered the membrane tail tube unnecessary. The packaging system of adenovirus would have consequently been adjusted to this new situation.

4.6 Minor capsid protein P6 is required for efficient DNA packaging (IV)

The observation that the minor capsid protein P6 is located at a single vertex (Gowen et al., 2003), and the dependence of P6 incorporation into capsids on proteins P20 and P22, suggested that P6 could play a role in DNA packaging. Additional support for the role of P6 in packaging came from the genome organisation of PRD1. In PRD1, as in many other viruses, genes coding for proteins functioning together and performing related functions are often located close to each other in the genome. Gene *VI*, coding for P6, is located in front of the packaging ATPase gene *IX*, and overlaps with gene *X*, which codes for the non-structural protein P10. P10 is involved in the formation virus-specific lipid vesicles, on top of which the PRD1 capsid proteins are assembled. This overlap, which is conserved in the tectiviral genomes, prompted us to suspect that P6 could also play a role in capsid assembly.

To clarify the role of P6 in virus assembly and DNA packaging, a suppressor-sensitive mutant defective in PRD1 gene *VI*, *sus621*, was isolated. EM analysis of *sus621*-infected cells and analysis of purified mutant particles revealed that capsid assembly was not affected, and particles of normal size and shape were produced. The total amount of particles produced by the P6-deficient mutant was

comparable to wt infection, as determined by both EM of infected cells and purification of the virus particles by sucrose gradient centrifugation. However, the subsequent stages of maturation were gravely impaired in the P6-deficient mutant, and mostly empty particles were produced. The lack of P6 did not lead to a total loss of packaging activity, as approximately 5 % of the progeny particles were found to be mature DNA-containing virions. However, the 95 % drop in the number of DNA-filled progeny particles strongly suggests a role for P6 in DNA packaging.

When the P6-deficient mutant particles were analysed in more detail by SDS-PAGE and Western blotting, no other structural proteins were found to be missing, but it was noticed that the DNA-filled particles contained less than wt amounts of packaging ATPase P9. Surprisingly, also the empty $P6^-$ particles contained some P9, although P9 is not a component of empty wt procapsids. Additionally, the empty $P6^-$ particles were found to contain DNA-binding protein P19, which is neither a component of empty nor DNA-filled particles in wt PRD1 infection. These results suggested that the empty Sus621 particles could be the products of unsuccessful packaging attempts.

The few DNA-filled particles produced in $P6^-$ infection were found to be stable and infective, although their specific infectivity was reduced 100-fold (although the particles were still 10-20 fold more infective than the preparation of empty particles). The DNA in the filled particles was confirmed by restriction enzyme digestion to be PRD1-specific, ruling out the possibility of P6 being a specificity-determining factor in PRD1 DNA packaging. The stability of the DNA-filled particles suggested that P6 was not a 'plug' either, preventing the leakage of DNA from packaged particles through the packaging vertex. Although the DNA was stably contained in the virions, the lack of P6 seemed to make the particles more accessible to small molecules, as judged by transmission electron microscopy (TEM) of negatively-stained purified virus particles, where stain penetration to the interior of both DNA-filled and empty P6-deficient particles was readily observed, in contrast to the situation in wt PRD1, where stain penetration into DNA-filled particles could not observed. The fact that P6 mutant particles were not as infective as wt virions could possibly be due to P6 actually playing a role in DNA entry into cells. However, a more plausible explanation is that in the absence of P6, DNA packaging is somehow incomplete. This would leave the DNA in an 'injection-incompetent' state in the virion, due to, for example, reduced internal capsid pressure or the DNA having an incorrect conformation.

The $P6^-$ particles were analysed for their ability to package DNA using the *in vitro* packaging system, but only very little packaging *in vitro* was observed, as compared to the standard assay with $P9^-$ particles, and only when additional packaging ATPase P9 was added. Taken together, the fact that some DNA packaging could be observed in cells infected with the P6-deficient mutant, with most progeny particles being empty, and that the P9 and P19 containing empty particles could not be efficiently packaged *in vitro*, suggests that the empty $P6^-$ particles are products of unsuccessful packaging attempts, essentially a dead-end pathway. Therefore we conclude that P6 is essential for efficient DNA packaging, and is probably needed either for correct and efficient binding of P9 and the viral DNA-terminal protein complex to the procapsid, and/or for stimulation of P9 activity.

Although comparisons to the DNA packaging systems of other dsDNA viruses revealed no obvious counterparts for P6, certain functional and sequence-level similarities could be observed between P6 and the lambda packaging factor gpFI and the adenovirus L4 22kDa protein. Lambda gpFI is involved in mediating terminase binding to the procapsid and in the initial cos-cleavage of the concatemeric DNA (Murialdo and Tzamtzis, 1997; Murialdo et al., 1997; Sippy and Feiss, 2004). This is somewhat similar to the case in PRD1 mutant, where P6 may be needed for binding packaging ATPase P9 to the procapsid, although obviously, no cos-type cleavage is necessary for the unit-length PRD1 genome. Both gpFI and P6 are very acidic

(theoretical pIs 4.6 and 4.0, respectively), similar in size, and have similar glutamate-rich sequences in their N-terminal half. The adenovirus L4 22 kDa protein is similarly rich in glutamate and has been shown to be involved in DNA binding and DNA encapsidation, together with the adenovirus IVa2 protein, which is another DNA packaging protein with putative ATPase activity (Zhang et al., 2001; Ostapchuk et al., 2006). Thus, the L4 22 kDa protein could play a similar role in adenovirus infection as P6 does in PRD1.

Both bacteriophage phi29 and PRD1 have similar unit-length genomes with covalently attached terminal proteins. In phi29, the structural pRNA plays an essential role in DNA packaging but in PRD1 no such RNA has been observed. Tentative functional parallels can however be drawn between PRD1 P6 and the pRNA molecules of phi29, as pRNA is needed for binding of phi29 ATPase gp16 to the procapsid and to stimulate the ATPase activity of gp16 (Grimes and Anderson, 1990).

In addition to the role of P6 in packaging, the mechanism of its incorporation into one vertex of the particle during assembly is another interesting question. In many dsDNA phages, portals and other minor capsid proteins present at only one vertex have been shown to interact with scaffolding proteins and the coat proteins, the ratio of the proteins being also important for correct incorporation of one portal per virion. The location of gene *VI* in the PRD1 genome in an overlapping reading frame with gene *X*, which codes for the non-structural protein P10 needed for vesicle formation, could provide a mechanism for controlling the expression of P6 in relation to P10, ensuring that the proteins are produced in a correct ratio and that P6 is incorporated into the forming capsid.

4.7 Model for PRD1 packaging

PRD1 has a unique packaging vertex, similar to what is seen in other dsDNA bacteriophages. However, the presence of the internal membrane in the PRD1 virion presents unique requirements on DNA packaging and the structure of a portal, as the PRD1 genome needs to be inserted into the lipid vesicle residing underneath the protein capsid. The solution to this challenge in PRD1 has been the use of two small membrane proteins, P20 and P22, as part of the packaging vertex structure, and probably these proteins provide the channel necessary for DNA passage through the lipid bilayer. Thus, the structure of the portal, and most probably also the mechanism of packaging, differ significantly from those of the tailed phages. Based on the findings in this study (I, III, IV) and earlier work on PRD1 structure and assembly, we propose a model for PRD1 DNA packaging. The model encompasses several features seen in the packaging of tailed dsDNA phages but clearly represents a different type of packaging system from those of the tailed phages and herpesviruses.

As the PRD1 genome is replicated as a unit-length molecule, there is no need for a terminase enzyme with both DNA translocation and cleavage activities, and therefore a single packaging ATPase, P9, protein is probably used. Packaging ATPase P9 was shown to be able to package DNA into preformed empty particles *in vitro*. Unlike the tailed phages and herpesvirus, where the terminase or packaging ATPases dissociate from the procapsid after packaging, PRD1 P9 stays attached to the mature virion. Interestingly, the putative B164 ATPase of the archaeal virus STIV has similarly been shown to be a component of the mature virion (Maaty et al., 2006), so this may be a common feature of the packaging ATPases of internal membrane-containing viruses.

PRD1 has a terminal protein (P8) covalently attached to the 5' ends of its linear genome, similar to what is seen in bacteriophage phi29. The phi29 terminal protein has been suggested to be equivalent to the DNA-binding small terminase subunits in viruses with two-component terminase enzymes packaging, and it has been shown to be important in DNA packaging. Similarly, we assume that the PRD1 terminal protein may play a role in DNA packaging, possibly by mediating DNA binding to the packaging machinery and by acting as a specificity-determining factor. A phi29 pRNA-type packaging RNA molecule however does not seem to be a component of the PRD1 packaging machinery.

We thus propose a model (see Fig.15.) were DNA packaging is initiated by binding of the P9 packaging ATPase to the DNA-P8 complex, and the P9-DNA-P8 complex then proceeds to bind the procapsid at the packaging vertex, analogous to the initiation of packaging in other dsDNA phages.

Another option would be that P9 binds to empty procapsids, which then bind DNA, but this is highly unlikely, as empty particles with P9 attached are never seen in wt infection, nor in mutants were no DNA packaging at all can be detected, such as in $P9^-$ or $P20^-$ mutants (I). Binding of the P9-DNA-P8 complex to the capsid is most probably mediated by P6 and/or the small membrane proteins P20 and P22, but probably not P6 alone, as in its absence some P9 can still be found in the virus particles, although the packaging efficiency is greatly reduced (IV). Alternatively, P6 may also act by directly stimulating the ATPase and DNA translocation activities of P9.

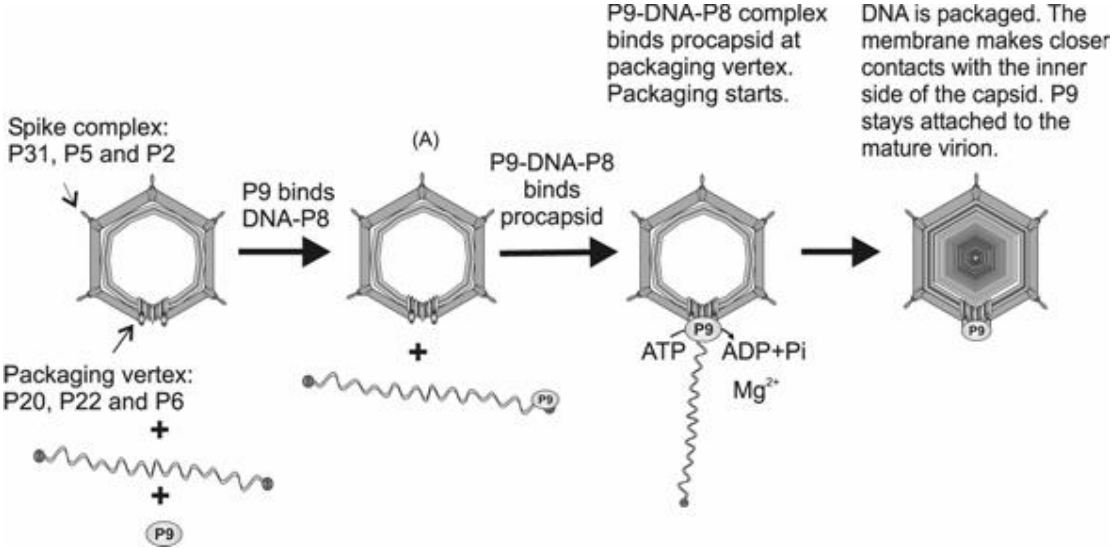

Figure 15. A model for PRD1 DNA packaging

After packaging, which is an ATP-dependent process, the packaging ATPase P9 stays attached to the capsid, possibly acting as a plug, sealing the packaged particles and preventing DNA release. The terminal protein P8 may also play a role in particle closure. Alternatively, the packaged DNA may cause a conformational change (for example in the small membrane proteins) sealing the particle and preventing premature DNA release, similar to the conformational change in the portal of P22 in response to increasing DNA pressure around the portal inside the capsid, as proposed by Lander et al (2006). No capsid expansion in relation to DNA packaging is seen in PRD1, but instead the internal membrane makes closer contacts with the inner side of the P3 protein capsid and adopts a more angular shape (Butcher et al., 1995; San Martín et al., 2001; San Martín et al., 2002). This membrane reorganisation is most probably triggered by interactions between the negatively charged DNA backbone and the lipid head groups and could well act as a part of a signal to the PRD1 packaging vertex proteins to trigger capsid sealing and packaging termination. An alternative, and perhaps more controversial, idea is that the membrane-embedded portal structure of PRD1 procapsids would be mainly in a ‘closed’ conformation, the presence of P9 and DNA only transiently opening it for packaging, and once the second terminal protein P8 is packaged, the structure ‘automatically’ returns to a closed conformation.

5 CONCLUSIONS AND FUTURE PROSPECTS

Bacteriophage PRD1 uses a unique assembly mechanism where capsid proteins are assembled not using protein scaffolding, as in many other dsDNA bacteriophages, but a membrane vesicle as a scaffold on top of which the capsid proteins are assembled, enclosing the membrane within a protein capsid. PRD1 was for long known to assemble empty precursor particles which were subsequently filled with DNA, but how this task was accomplished was not understood, as there was no evidence for a unique packaging vertex similar to for example the case in tailed phages. The presence of the internal membrane further complicated the picture, as it would be necessary for the DNA to be transported across both protein and lipid layers.

In this study, packaging-related proteins of PRD1 were identified and shown to be located at a single vertex of the PRD1 virion, independent from the receptor-binding common vertex complex at the other vertices of the virion. This unique packaging vertex of a non-tailed bacteriophage was the first of its kind to be identified. An *in vitro* system was developed, which allowed a more detailed analysis of the components involved in the DNA packaging mechanism of PRD1, the first such a system for a virus containing an internal membrane. The roles of the individual packaging proteins P20, P22, P6 and P9 were studied, and a model for PRD1 packaging was proposed.

Sequence analysis and database searches revealed the presence of PRD1 P9-type putative packaging ATPases in viruses infecting host organisms in all three domains of life, Bacteria, Eukarya and Archaea. A new packaging ATPase motif was identified, clearly distinguishing the membrane-type ATPases from the packaging ATPases of other types of dsDNA viruses, for example the tailed phages or herpesviruses. The finding of a similar type of putative ATPase sequences in a group of mostly icosahedral, membrane-containing viruses infecting hosts of all domains of life, suggests that these viruses may share a common packaging mechanism using a unique packaging vertex and a membrane-type packaging ATPase. Not only may the PRD1-type packaging system represent a new class of genome packaging mechanisms, the knowledge obtained on the PRD1 system may thus help in understanding the principles of DNA packaging in viruses of the Eukarya and Archaea as well, among them several viruses pathogenic to humans and animals.

The finding of similar packaging proteins in almost exactly the same group of viruses previously found to have similar coat protein structures supports the suggestion that these viruses may share a common ancestor, possibly even predating the division of Bacteria, Eukarya and Archaea. The accumulation of more structural and sequence information will hopefully help in further clarifying the evolutionary relationships of this group of viruses.

The refinement of the *in vitro* packaging system to include purified viral particles and P9, instead of cell extracts, will hopefully help in solving many of the remaining questions on the mechanism of DNA packaging in PRD1. An important question is if any non-structural viral or host proteins are involved in packaging. Whether P8 is actually needed in packaging is also still unclear, as well as the exact binding interactions between the different packaging proteins and/or DNA. Additionally, such a refined *in vitro* system could possibly be applied to single-molecule studies, providing even more detailed information on the DNA packaging mechanism, such as the forces created by the packaging motor. The set of P9 mutant proteins created in this study will provide ample material for further biochemical analyses characterising the ATP hydrolysis and other possible activities of P9.

As the packaging proteins are located at a single vertex of the virion, they cannot be seen in icosahedrally averaged structures of the virion. Obtaining the X-ray structures of the individual packaging proteins and maybe even an asymmetric cryo-EM structure of the whole virion with the packaging vertex visible, would greatly help in understanding the structure of the packaging vertex and the role of each protein in PRD1 DNA packaging. No P9-type packaging ATPase protein structure has yet been solved by X-ray crystallography. Therefore structural studies would be invaluable not for PRD1 alone, but for studies on other membrane-type ATPases as well.

One of the most interesting questions is whether the packaging vertex is also used for DNA injection. If so, this could point to a dual-receptor model: first primary binding to the host cell would occur via the P2-receptor binding protein of the common vertex complex, and then the virion would roll over to the single packaging/ejection vertex to initiate DNA ejection into the cell. Alternatively, it is possible that although packaging occurs through a single vertex, ejection could occur through any of the other 11 vertices. In such case, the organisation of the genome within the virion is of specific interest, as the genome should be positioned in an ejection competent way in relation to all the possible ejection vertices. However, the fact that the PRD1 genome is injected inside the membrane tail tube, and not as naked DNA, may provide the means for such an ejection mechanism.

The work presented here, and especially the developed *in vitro* packaging assay, provides a basis for the further analysis of DNA packaging in PRD1. Not much is known about viruses containing internal membranes and even less about their DNA packaging mechanisms. Hopefully, this work with PRD1 has now opened up new possibilities for future experiments and can provide people with tools for understanding DNA packaging not only in PRD1, but in other internal membrane viruses as well.

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The Unique Vertex of Bacterial Virus PRD1 Is Connected to the Viral Internal Membrane

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Icosahedral double-stranded DNA (dsDNA) bacterial viruses are known to package their genomes into preformed procapsids via a unique portal vertex. Bacteriophage PRD1 differs from the more commonly known icosahedral dsDNA phages in that it contains an internal lipid membrane. The packaging of PRD1 is known to proceed via preformed empty capsids. Now, a unique vertex has been shown to exist in PRD1. We show in this study that this unique vertex extends to the virus internal membrane via two integral membrane proteins, P20 and P22. These small membrane proteins are necessary for the binding of the putative packaging ATPase P9, via another capsid protein, P6, to the virus particle.

The genome packaging of icosahedral double-stranded DNA (dsDNA) bacteriophages occurs by translocation of the genome into preformed procapsids. This translocation is performed by a specific enzyme, a terminase or a packaging ATPase, and is powered by ATP hydrolysis. The packaging of DNA occurs at a unique portal vertex, which also functions as the head-to-tail connector as well as the channel through which DNA is injected into the host cell during infection. The portals characterized thus far are ring-like structures of portal protein multimers with a central channel correctly sized for the dsDNA to be threaded through. Packaging is believed to occur by winding the DNA up through this central channel, involving movement of the portal structure (16, 20, 35, 42, 43, 64, 66). A symmetry mismatch between the packaging machinery and the rest of the capsid has been proposed to assist the movement of the packaging machinery with respect to the capsid during nucleic acid transport (35, 42, 64).

The most detailed description of a packaging system exists for bacterial virus ϕ29, a short-tailed icosahedral prolate virus with a linear dsDNA genome, infecting the gram-positive *Bacillus subtilis* (2). The ϕ29 portal occupying a pentagonal vertex is a dodecamer of portal protein gp10, forming a propeller-shaped structure with a central channel (35, 64). In addition to the portal, which is attached to the prohead, the packaging machinery consists of five or six copies of the viral ATPase (gp16) and six copies of a ϕ29-encoded packaging RNA (pRNA) (36–39, 74). The DNA, connector, and prohead-pRNA-ATPase complex form a set of concentric structures with 10-, 12-, and 5- or 6-fold symmetry, respectively, embedded in the 5-fold vertex of the prohead (35, 64).

A portal assembly complex has also been identified in a eukaryotic virus. The portal of herpes simplex virus type 1 (HSV-1), identified by immunogold labeling, is located at a single vertex of the icosahedral HSV-1 capsid and contains a ring-shaped multimer of the UL6 protein (52). So far, other portal vertices of icosahedral eukaryotic viruses have not been described. It is conceivable, however, that many more complex icosahedral viruses assemble through packaging of empty precursor particles, which would require a unique portal complex.

PRD1 is the type organism for the family *Tectiviridae* (7). It is a bacterial virus that infects a variety of gram-negative hosts harboring an N-, P-, or W-type conjugative plasmid (54). The PRD1 virion consists of an icosahedral protein capsid surrounding an internal membrane that encloses a 14,927-bp linear dsDNA genome with inverted terminal repeat sequences at both termini and covalently linked 5′-terminal proteins (5, 6, 12, 62). DNA replication of PRD1 is initiated by protein priming from the terminal proteins (27, 28, 63). The PRD1 membrane is comprised of approximately half lipid and half protein (12, 31). The lipids are derived from the plasma membrane of the host cell, and the proteins are encoded by the viral genome (12, 31). During infection, the spherical membrane vesicle undergoes a structural transformation into a tubular tail-like structure that is thought to inject the genome into the host cell (4, 34, 46, 56).

Structural information from cryoelectron microscopy and image processing as well as X-ray crystallography has shown that PRD1 is surprisingly similar to adenovirus (17–19, 26, 60, 61). Similar to the organization of the adenovirus capsid (24), the capsid of PRD1 is organized in a pseudo-$T=25$ lattice with 240 copies of the trimeric coat protein P3, an arrangement that has not been observed in any other virus (26). In addition, the fold of the PRD1 major capsid protein P3 closely resembles that of the adenovirus coat protein (18, 19). The PRD1 capsid is stabilized by a glue protein, P30 (57), and is further stabilized by the N and C termini of the major coat protein (61), analogous to the four different species of cementing proteins stabilizing the capsid of adenovirus (25). The vertex structures of the two viruses are also very similar. Analogous to the penton-spike complex of adenovirus, the PRD1 vertex structure is comprised of the pentameric penton base protein P31 and the trimeric spike protein P5 (13, 29, 55, 65). In PRD1, a third protein species, the receptor-binding protein P2, is the functional analogue of the knob domain of the adenovirus spike (32, 55, 71, 72). The PRD1 P31$^-$ mutant lacks the entire spike complex and the peripentonal coat protein trimers, and large openings can be seen at the vertices of the virus particle (55).

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TABLE 1. Bacterial strains used in this study

Strain	Description	Relevant property	Reference(s)
S. enterica serovar Typhimurium LT2			
DS88	SL5676 ΔH2 H1-i::Tn*10* (Tc^s) *non rev* (pLM2)	Nonsuppressor host	10
PSA(pLM2)	*supE*	Suppressor host for *sus400*	47
DB7154(pLM2)	DB700 *leuA141*(Am) *hisC527*(Am) *supD10*	Suppressor host for *sus526*	70
DB7156(pLM2)	DB700 *leuA141*(Am) *hisC527*(Am) *supF30*	Suppressor host	70
E. coli K-12			
HMS174	*recA1 hsdR* Rif^r		30
HB101	*supE44 hsdS20*(r_B^- m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Cloning host	21, 22
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Cloning host	40, 59

Translocation of the PRD1 genome into the capsid probably is performed by the putative packaging ATPase P9 (12), since in its absence only empty virus particles are produced (49). Unlike packaging ATPases of most other icosahedral dsDNA bacteriophages, P9 is a structural protein (49). In addition, PRD1 has many other structural proteins, which are either located within or attached to the internal membrane (14). P6 is a minor capsid protein, the function of which is still unclear (48). Small integral membrane proteins P20 and P22 are known to be involved in DNA packaging or in the stable maintenance of the DNA within the particle (49). Proteins P7, P14, P11, P16, P18, and P32 are all part of the DNA delivery apparatus (33, 34). Protein P11 has been proposed to be necessary for penetrating the bacterial outer membrane and making the peptidoglycan layer accessible for P7, the viral transglycosylase (34, 56). The delivery process is then continued by formation of the membrane tail tube structure in which at least proteins P14, P16, P18, and P32 are involved (4, 33, 34). Lysis of host cells is performed by P15, a lytic muramidase (58).

Although most icosahedral dsDNA bacterial viruses contain a unique vertex, which is used for DNA packaging and injection (20, 43), no such vertex has yet been shown to exist in PRD1. The structural methods used in analysis of PRD1 architecture are based on icosahedral averaging. This excludes any asymmetrically located structures, e.g., at a single vertex. Very recently, immunogold labeling has provided the first evidence of a unique vertex in PRD1 (B. Gowen, J. K. H. Bamford, D. H. Bamford, and S. D. Fuller, submitted for publication). Gold-conjugated P6- and P20-specific antibodies were shown to bind to a single vertex of PRD1. This suggested that at least the minor capsid protein P6, which has no previously appointed function, and the membrane protein P20 are located at a unique vertex.

During the years of research on PRD1, mutants with amber mutations in different PRD1 genes have been isolated (13, 32, 33, 48, 55, 56, 61). A thorough analysis of all available PRD1 mutant particles with all available antibodies against structural proteins was performed in this study. Special emphasis was given to mutants with mutations in the spike complex proteins P31, P5, and P2 and to mutants with defects in DNA packaging or stabilization of packaged particles.

MATERIALS AND METHODS

Bacteria and phages. Bacterial strains and viruses used in this study are listed in Tables 1 and 2, respectively. Cells were grown at 37°C in Luria-Bertani (LB) medium (59), and when appropriate, chloramphenicol (25 μg/ml) was added. Wild-type (wt) PRD1 was propagated on *Salmonella enterica* serovar Typhimurium LT2 DS88 and its mutant derivatives on suppressor strains PSA(pLM2), DB7154(pLM2), and DB7156(pLM2). PRD1 amber mutants were induced with *N*-methyl-*N*′-nitrosoguanidine and isolated as described previously (48).

For production of wt and mutant PRD1 virus particles, DS88 cells were infected at a multiplicity of infection of 6 and 8, respectively. After lysis of the cells, phage particles were purified by polyethylene glycol-NaCl precipitation and 5 to 20% rate-zonal sucrose gradient centrifugation, as previously described (10). The virus was concentrated by either differential centrifugation or further purified by ion-exchange chromatography on Sartorius D100 anion-exchange cartridges, essentially by the method of Walin et al. (69).

DNA techniques. Plasmids used in this study are listed in Table 2. DNA manipulations were done using standard molecular biology techniques (59). For the complementation analysis of the PRD1 mutants, PRD1 genes *IX* and *XXII*, together with their preceding ribosome binding site sequences, were amplified by PCR using primers specific to the corresponding genes in the PRD1 genome, and the resulting fragments were inserted between the *Eco*RI and *Hin*dIII sites of

TABLE 2. Bacteriophages and plasmids used in this study

Bacteriophage or plasmid	Description	Reference(s) or source
PRD1		
wt	wt	54
sus1	Amber mutation in gene *IX*, two missense mutations in gene *VI*	48
sus42	Amber mutation in gene *XXII*	48
sus148	Amber mutation in gene *XVIII*	48
sus234	Amber mutation in genes *VII* and *XIV*	48
sus400	Amber mutation in gene *XX*	This study
sus471	Amber mutation in gene *II*, opal mutation in gene *VII* ($P2^-$/$P7^-$ when propagated in nonsuppressor host, $P7^-$ in suppressor host)	56
sus525	Amber mutation in gene *XXXI*	55
sus526	Amber mutation in gene *XX*	This study
sus539	Amber mutation in gene *II*	13, 55
sus607	Amber mutation in gene *XI*	61
sus690	Amber mutation in gene *V*	13
[*lacZ*α]-9	Amber mutation in gene *XXXII*	33
Plasmids		
pSU18	Cloning vector, p15A replicon	15
pNS1	PRD1 gene *IX* (nt 7624–8320)[a] in pSU18	This study
pMG119	PRD1 gene *XX* (nt 8445–8588) in pSU18	Marika Grahn
pMV11	PRD1 gene *XXII* (nt 9784–9944) in pSU18	This study
pLM2	Encodes the PRD1 receptor	47

[a] Numbers refer to the PRD1 genome sequence (GenBank accession number M69077).

plasmid pSU18. The constructs were transformed into *Escherichia coli* HB101 or DH5α cells. The DNA sequences of the inserts were determined, and the constructs were transformed into *E. coli* HMS174(pLM2). The exact locations of the amber mutations in the PRD1 mutants were determined by sequencing the region from nucleotides (nt) 6784 to 9944 from each of the mutants. Overlapping PCR fragments amplified with primers specific to the PRD1 genome were used as templates in the DNA sequencing reaction. Purified virus DNA (in the case of *sus1* and *sus42*) or viral DNA from plaques (45) (in the case of *sus400* and *sus526*) was used as a template in the PCRs. Sequencing was performed using an automated sequencer (at the DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, University of Helsinki).

Electron microscopy. For thin-section electron microscopy, DS88 cells were grown in LB to a density of 10^9 CFU/ml and infected with *sus400* and *sus526* PRD1 mutants using a multiplicity of infection of 8. Samples were taken 40, 60, and 70 min postinfection and fixed with 3% glutaraldehyde (vol/vol) in 20 mM potassium phosphate buffer (pH 7.2) for 20 min at room temperature. The cells were collected by centrifugation, washed twice with 20 mM potassium phosphate buffer (pH 7.2), and prepared for transmission electron microscopy as described previously (3). Electron micrographs were taken with a JEOL 1200 CX microscope (at the electron microscopy unit, Institute of Biotechnology, University of Helsinki) operating at 60 kV.

Polyclonal sera. Polyclonal sera against proteins P9 and P22 were raised in rabbits using specific peptides as antigens. The peptides CTADAVLARFDLG KKR, corresponding to residues 209 to 223 of P9, and MQLITDMAEWSSKPC, corresponding to residues 1 to 14 of P22, were purchased from KJ Ross-Petersen AS, Copenhagen, Denmark. The peptides were linked to keyhole limpet hemocyanin via the cysteine residue. Rabbits were immunized three times at 3-week intervals using the protein-conjugated specific peptides emulsified with either Freund's complete adjuvant (in the first immunization) or incomplete adjuvant (in the subsequent immunizations). One milligram of the conjugated peptide was used per immunization. The specificity of the serum obtained was determined by Western blotting.

Analytical methods. The protein concentration of purified virus preparations was determined by the Coomassie brilliant blue method using bovine serum albumin as a standard (23). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (53). Western blotting was performed by transferring the proteins from SDS-polyacrylamide gels (17% polyacrylamide) onto polyvinylidene difluoride membranes (Millipore). Polyclonal antisera against PRD1 proteins P2 (32), P5 (41), P31 (55), P9 and P22, together with monoclonal antibodies 6T58, 7A5, 7N41 (41), and 11A401 (Gowen et al., submitted) against proteins P6, P7/P14, P7/P14, P16, and P11, respectively, were used as primary antibodies. Proteins were visualized using the Supersignal West Pico chemiluminescent substrate (Pierce) with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin G (IgG) antibodies (Dako) or peroxidase-labeled horse anti-mouse IgG antibodies (Vector) as secondary antibodies.

RESULTS

Isolation and identification of PRD1 mutants with amber mutations in gene *XX*. Two suppressor-sensitive gene XX^- mutants of PRD1, *sus30* and *sus78*, have been described previously (48). However, when analyzed by DNA sequencing, these mutants did not contain mutations in gene *XX,* but instead, the mutations were in several other genes (data not shown). To obtain genuine gene *XX* mutants, *N*-methyl-*N*′-nitrosoguanidine mutagenesis was performed on PRD1 and new amber mutants were selected. Mutations were provisionally mapped to particular regions of the genome by marker rescue analysis (12, 50). Two new amber mutants were obtained and subsequently named *sus400* and *sus526*.

DNA sequencing reveals amber mutations in genes *IX*, *XXII*, and *XX*. The exact defects in PRD1 packaging mutants, first preliminarily mapped by marker rescue analysis to the region of the genome containing genes *VI*, *X*, *IX*, *XX*, and *XXII* were identified by DNA sequencing of the genome region from nt 6784 to 9944 of the mutants (Fig. 1). The mutations in *sus400* and *sus526* were located at nt 8470 (G→A) and 8497(G→A),

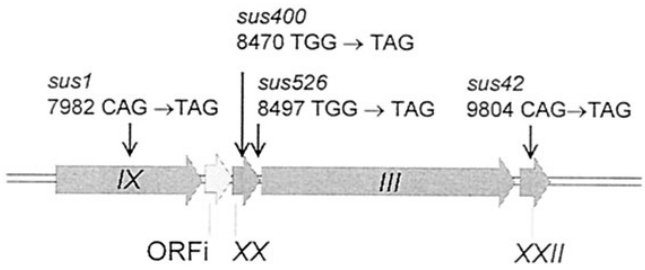

FIG. 1. Sequencing of the region from nt 6784 to 9944 of the PRD1 genome that contains genes *IX*, *XX*, and *XXII*. Gene *III* encodes the major capsid protein. The function of open reading frame ORFi is unknown. Defects found in different suppressor-sensitive mutants are marked with arrows.

respectively. Both mutations result in amber codons and fall within the sequence of gene *XX*, yielding 3- and 12-amino-acid-long amber fragments for *sus400* and *sus526*, respectively. *sus42* contained a transversion at nt 9804 (C→T), resulting in introduction of an amber mutation at the second codon of gene *XXII*. *sus1* contained an amber mutation at nt 7982 (CAG→TAG) within gene *IX*, yielding a 115-amino-acid-long amber fragment instead of the 227-amino-acid-long wt P9. Thus, we confirm that *sus1* and *sus42* contain amber mutations in genes *IX* and *XXII*, respectively, as had been proposed previously (48).

The mutations can be complemented by a single gene. To ensure that the amber mutations identified by DNA sequencing were the only relevant mutations affecting the mutant viruses, complementation analysis was performed. In the complementation assay, the titers of PRD1 mutants were determined on *E. coli* K-12 HMS174(pLM2) strain harboring either vector pSU18 or recombinant plasmid pNS1, pMV11, or pMG119 containing a single wt virus gene in pSU18. Overexpressed wt proteins P9 (pNS1) and P22 (pMV11) were found to fully complement the defects in *sus1* and *sus42*, respectively (Table 3). The defects in both *sus400* and *sus526* were corrected by P20, produced from pMG119 (Table 3), further confirming that *sus400* and *sus526* have mutations in gene *XX*. The titers of the mutant viruses on the complementing strains were, in all cases, equivalent to the titers obtained with wt virus. This confirms that these mutants do not have other defects affecting the viability of the viruses.

$P9^-$, $P20^-$, and $P22^-$ PRD1 mutants produce empty virus particles. PRD1 mutant and wt particles were grown in DS88 cells and purified by rate-zonal centrifugation. In the case of wt virus, two light-scattering zones, corresponding to packaged (80%) and empty (20%) virus particles, are observed in sucrose gradients as determined by using radioactively labeled virus particles and fractionation (11, 49). When *sus1*, *sus42*, *sus400*, and *sus526* mutants were purified by sucrose gradients, a clear light-scattering zone, corresponding to empty particles, could be seen in all cases. Another, albeit weak, zone corresponding to packaged particles, was visible in the case of *sus42*. This mutant has previously been demonstrated to package DNA, as determined by thin-section electron micrographs of infected cells (49). However, the DNA was lost upon subsequent purification. When *sus400* and *sus526* PRD1 mutants were analyzed by thin-section electron microscopy, only empty

TABLE 3. Complementation titers of gene *IX*$^-$, *XX*$^-$, and *XXII*$^-$ mutants

Strain	Property	Titer (PFU/ml)			
		sus1 (*IX*$^-$)	*sus400* (*XX*$^-$)	*sus526* (*XX*$^-$)	*sus42* (*XXII*$^-$)
S. enterica					
DS88	Nonsuppressor	4.4×10^4	4.5×10^5	4.0×10^7	3.4×10^6
PSA	Suppressor	3.5×10^{11}	8.0×10^{11}		1.5×10^{12}
DB7154	Suppressor			1.0×10^{12}	
E. coli					
HMS174(pLM2)					
HMS174(pSU18)	Negative control	1.0×10^3	1.2×10^5	3.8×10^6	3.3×10^5
HMS174(pNS1)	Gene *IX* in pSU18	1.0×10^{11}			
HMS174(pMG119)	Gene *XX* in pSU18		3.3×10^{11}	2.4×10^{11}	
HMS174(pMV11)	Gene *XXII* in pSU18				3.4×10^{11}

virus particles were seen in samples of infected cells taken 40, 60, and 70 min postinfection (Fig. 2). This was also the case for *sus1* (49).

Western blot analysis of the purified mutant particles. To analyze the phenotypic effects caused by different nonsense mutations, the protein content of different PRD1 mutant particles (P2$^-$, P5$^-$, P7$^-$, P7$^-$/P14$^-$, P9$^-$, P11$^-$, P18$^-$, P20$^-$, P22$^-$, P31$^-$, and P32$^-$) was assayed using Western blotting with all available PRD1-specific antibodies against structural proteins (see Materials and Methods). Unfortunately, the previously obtained anti-P20 serum (Gowen et al., submitted) did not give any signal on Western blots. Polyclonal sera against the putative packaging ATPase P9 and the membrane protein P22 were obtained by immunization of rabbits with keyhole limpet hemocyanin-conjugated P9- and P22-specific peptides.

It has been previously shown that mutant particles lacking the vertex protein P31 (*sus525*) are also devoid of the spike proteins P5 and P2 and of the peripentonal trimers, thus displaying large openings at the vertices (55). However, we show here by Western blotting that these particles contain proteins P6 and P22. P6 and P20 have been shown by electron microscopy immunolabeling to be located at a single vertex (Gowen et al., submitted), indicating that 1 of the 12 vertices is different. Western blotting also revealed that the putative packaging ATPase P9 and the small membrane protein P22 were present in P31$^-$ particles (*sus525*) (Table 4 and Fig. 3). Further, when mutant particles lacking spike protein P2 or P5 were examined, stoichiometric amounts of proteins P9, P6, and P22 were present (Table 4).

Interestingly, all of the packaging mutant particles (P9$^-$, P20$^-$, or P22$^-$) were shown to contain a full complement of spike complex proteins P31, P5, and P2 (Table 4). There was no mutant available for protein P6, but intriguingly P20$^-$ and P22$^-$ mutant particles were found to lack both proteins P6 and P9 (Table 4 and Fig. 3, which contains representative Western blots.) The expression levels of proteins P6 and P9 in cells infected with P20$^-$ and P22$^-$ mutants were analyzed by Western blotting, and their levels were equivalent to those found in wt virus infection (Fig. 4). Thus, the absence of P6 and P9 in the purified virus particles was not due to reduced production of these proteins in infected cells. None of the other PRD1 mutant particles analyzed (P7$^-$, P7$^-$/P14$^-$, P11$^-$, P18$^-$, P31$^-$, and P32$^-$) was found to lack any of the spike complex or packaging proteins. Furthermore, none of the other proteins analyzed (P11, P7, P14, and P16) by specific antibodies was linked to the loss of P6, P9, P20, P22, or the spike complex proteins (Table 4). In *sus1* particles (defect in gene *IX*), the only protein missing was P9.

The unique vertex is linked to the membrane. All mutants lacking either one of the small membrane proteins P20 and P22 were devoid of P6. Furthermore, in the absence of P6, none of the mutants tested contained P9. This suggests that the small integral membrane proteins P20 and P22 are attached directly or indirectly, probably via protein-protein interactions, to protein P6, which in turn is associated with protein P9. This series of associations, together with the fact that both the integral membrane protein P20 and the minor capsid protein P6 have been localized at a single vertex (Gowen et al., submitted), suggests that P9 and P22 are located at the same vertex. Thus, we can conclude that PRD1 has a unique vertex, which contains the proteins involved in DNA packaging and/or maintenance within the particle and which extends to the phage internal membrane.

FIG. 2. PRD1 P20$^-$ mutants produce only empty virus particles. As an example, an electron micrograph of thin-sectioned DS88 cells infected with mutant *sus400* collected 60 min postinfection is shown. Bar, 500 nm.

DISCUSSION

In all icosahedral dsDNA bacteriophages studied so far, packaging occurs by translocation of the genome into preformed procapsids via a unique portal vertex that is also the

TABLE 4. Analysis of the protein composition of different PRD1 mutant particles by Western blotting

Genotype	Mutant	Protein composition[a]									
		P9 (DNA-packaging ATPase)	P6 (minor capsid protein, packaging)	P22 (DNA packaging) (M)	P2 (receptor-binding protein)	P5 (spike protein)	P31 (penton protein)	P7 (Transglycosylase) (M)	P14 (DNA delivery) (M)	P11 (DNA delivery)	P16 (infectivity) (M)
wt		+	+	+	+	+	+	+	+	+	+
wt (E)[b]		−	+	+	+	+	+	+	+	+	+
IX$^-$	*sus1*	−	+	+	+	+	+	+	+	+	+
XXII$^-$	*sus42*	−	−	−	+	+	+	+	+	+	+
II$^-$	*sus539*	+	+	+	−	+	+	+	+	+	+
V$^-$	*sus690*	+	+	+	−	−	+	+	+	+	+
XXXI$^-$	*sus525*	+	+	+	−	−	−	+	+	+	+
VII/XIV$^-$	*sus234*	+	+	+	+	+	+	−	−	+	+
VII$^-$	*sus471*	+	+	+	+	+	+	−	+	+	+
XI$^-$	*sus607*	+	+	+	+	+	+	+	+	−	+
XVIII$^-$	*sus148*	+	+	+	+	+	+	+	+	+	+
XX$^-$	*sus400*	−[c]	−	+	+	+	+	+	+	+	+
XX$^-$	*sus526*	−	−	+	+	+	+	+	+	+	+
XXXII$^-$	[*lac*Zα]-9	+	+	+	+	+	+	+	+	+	+

[a] The presence (+) or absence (−) of the indicated proteins in PRD1 mutant particles is given. (M), integral membrane protein based on transmembrane helix prediction and location in the viral membrane.
[b] (E), empty particles.
[c] (−), a small amount could be detected.

site of tail assembly (and thus is easily visualized). A unique vertex has also been found in a complex dsDNA animal virus, HSV-1, which may be evidence for the conservation of a basic mechanism of DNA packaging in viruses. In addition, we have shown here that the internal membrane-containing icosahedral dsDNA bacteriophage PRD1 has a unique vertex.

The unique vertex of PRD1 comprises at least the putative packaging ATPase P9, minor capsid protein P6, and two small membrane proteins P20 and P22, which are all sequentially associated, probably via protein-protein interactions, with each other. The location of P6 and P20 at the unique vertex has been confirmed by immunogold labeling with specific antibodies (Gowen et al., submitted). Interestingly, the unique vertex of PRD1 extends to the phage membrane via the integral membrane proteins P20 and P22. How P20 and P22 are located with respect to each other in the virus is not clear: P20$^-$ mutant particles do not lack the protein P22, so association of P22 with the virus capsid cannot be dependent, at least solely, on protein P20. The need for P22 for the association of P20 with the virus could not be proven, as a specific antibody against P20 on Western blots was not available. P6 was missing in both P20$^-$ and P22$^-$ mutants, suggesting that both of the proteins are necessary for P6 binding, either directly or via some other protein species.

The PRD1 spike complex, comprised of the penton base P31, the spike P5, and the receptor-binding P2 proteins, is probably located at all vertices (55). The fact that all of the packaging proteins identified thus far can be found in the mutant virus particle (*sus525*) missing the spike complexes and the peripentonal P3 trimers suggests that the unique vertex is functionally and structurally distinct from the spike complex. It is not known if the unique vertex of PRD1 contains the spike complex proteins in addition to the packaging machinery or if the spike complex actually occupies only 11 of the 12 vertices, the twelfth vertex being reserved for the packaging machinery and possibly for other unique vertex proteins.

The identification of a unique vertex in PRD1 will be a valuable tool for solving the structure of the whole virus in more detail, thereby revealing more of the functional structures of PRD1. Current methods used for structural analysis, such as X-ray diffraction and cryoelectron microscopy combined with image reconstruction, are based on averaging of the icosahedral data, which will, of course, average out the density from asymmetrically located protein species. On the basis of structural data, only a few of the 18 or more PRD1 structural proteins have been allocated exact positions in the virion. We believe that many of these proteins might be found at the unique vertex. The DNA delivery protein P11 could not be located by difference imaging of quasi-atomic models of cryoelectron microscopy reconstructions of P11$^-$, P9$^-$, and wt particles, suggesting that the distribution of P11 does not follow icosahedral symmetry (61). If P11 were located at the unique vertex, it would seem logical for the other proteins involved in DNA entry (P7 and P14) also to be situated at the same vertex. It has been proposed that association of the lysis protein P15 with the virus capsid is dependent on the small membrane protein P22 (58). Importantly, P22 was shown in this study to be part of the unique vertex, leading to the hypothesis that P15 is yet another unique vertex protein.

The striking structural and functional similarities found between adenovirus (which infects members of the domain *Eucarya*) and PRD1 (which infects members of the domain *Bacteria*) have led to the hypothesis that these viruses share a common ancestor and further that viruses may form lineages that have members infecting hosts in different domains of life (8, 9, 18, 44). This lineage may contain viruses of other evolutionarily distant hosts as well. For example, the same coat protein fold found in PRD1 and adenovirus can also be observed in Paramecium bursaria chlorella virus 1 (PBCV-1) (51), another internal-membrane-containing virus which infects unicellular, eukaryotic, chlorella-like green algae (67, 68, 73). Also, the genome organization and predicted coat protein

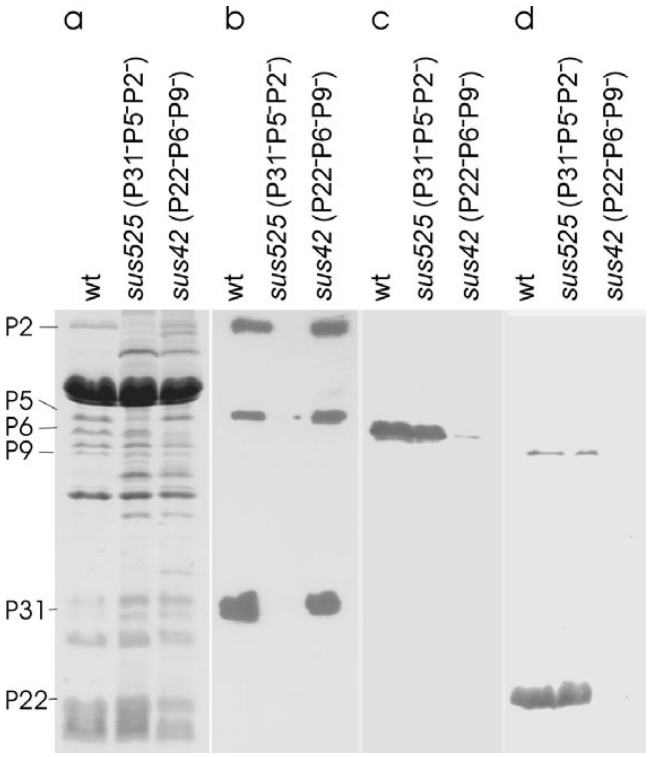

FIG. 3. Protein composition of purified virus particles of wt PRD1, spike complex mutant *sus525* (*XXXI*$^-$), and the small membrane protein mutant *sus42* (*XXII*$^-$) analyzed by Coomassie brilliant blue-stained SDS-PAGE (a) and Western blotting (b to d). For Western blotting, polyclonal sera against P2, P5, and P31 (b), monoclonal antibody against P6 (c), and polyclonal sera against P9 and P22 (d) were used for detection. The positions of proteins of interest are indicated to the left of the gel.

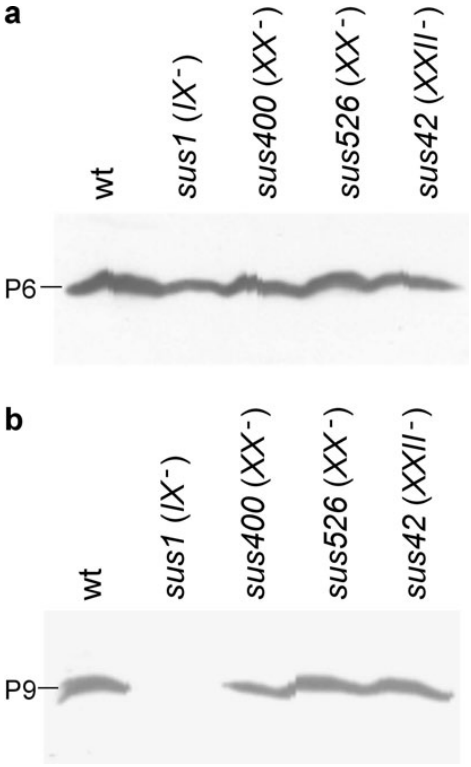

FIG. 4. Western blotting of cells infected with wt PRD1 (positive control), packaging mutant *sus1* (*IX*$^-$, negative control for P9 expression), and membrane protein mutants *sus400* (*XX*$^-$), *sus526* (*XX*$^-$), and *sus42* (*XXII*$^-$). (a) P6. (b) P9.

fold of bacteriophage *B*am35, which infects the gram-positive bacterium *Bacillus thuringiensis* (1, 7) are similar to those of PRD1 (54a).

So far, a unique vertex has been identified in only one icosahedral dsDNA virus infecting higher organisms, HSV-1 (52). As DNA packaging generally is regarded as a more conserved function and not as susceptible to evolutionary pressure as DNA entry-related mechanisms (9), it is enticing to hypothesize that although the hosts of PRD1, adenovirus, PBCV-1, and *B*am35, are evolutionarily far apart, their DNA packaging mechanisms may resemble each other. PBCV-1 has been observed to form empty precursor capsids and to inject its DNA into the host cell while leaving the empty capsid bound to the cell surface (68), in a fashion similar to PRD1. Both adenovirus and PBCV-1 contain structural proteins whose location and function in the virion are still poorly characterized (25, 68). It is conceivable that these viruses also possess a unique vertex for packaging, and thus studies on a bacterial virus are paving the way to understanding such properties in the families *Adenoviridae* and *Phycodnaviridae*.

It has been proposed that PRD1 could use any of its vertices for binding to the host cell and for DNA injection (4, 34, 46, 56). This is in contrast to the tailed dsDNA bacteriophages in which the attachment and injection machinery are found only at one vertex, attached to the portal, or connector, through which the DNA has been packaged. Even though the PRD1 receptor-binding protein P2 can be found at all or at least the majority of vertices (32, 55), it has not been shown directly whether DNA injection occurs in vivo through any of the vertices or if a certain vertex is preferred. The finding of a unique vertex in PRD1 points to the possibility that its DNA injection might actually occur through a single vertex. We propose a mechanism whereby PRD1 would make primary contact with the cell and bind reversibly to the host via P2 but then roll over so that the unique vertex could be reached, leading to irreversible binding and DNA injection. This model is supported by the flexibility of the spike protein P5 (45), which would enable the proposed rolling movement.

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The *Bacillus thuringiensis* Linear Double-Stranded DNA Phage Bam35, Which Is Highly Similar to the *Bacillus cereus* Linear Plasmid pBClin15, Has a Prophage State

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Bam35, a 15-kbp double-stranded DNA phage, infects *Bacillus thuringiensis*. Recently, sequencing of the related *Bacillus cereus* revealed a 15.1-kbp linear plasmid, pBClin15. We show that pBClin15 closely resembles Bam35 and demonstrate conversion of Bam35 to a prophage. This state is common, as several *B. thuringiensis* strains release Bam35-related viruses.

Phage Bam35 was originally isolated from *Bacillus thuringiensis* var. *alesti* strain 35 (1). As Bam35 could also be isolated from Bam35-resistant colonies of *Bacillus megaterium* and from a sensitive strain of *B. thuringiensis* var. *entomocidus*, a carrier state has been proposed (1). Recently, a clear-plaque mutant (Bam35c) was sequenced and further characterized (25). This work showed that Bam35 is related to PRD1, a linear double-stranded DNA virus with 5′-terminal proteins and an internal lipid membrane (4). PRD1 infects gram-negative bacteria harboring an IncP, -W, or -N conjugative plasmid. Structural analyses of PRD1 have, surprisingly, indicated that this member of the *Tectiviridae* is similar to the *Adenoviridae*, *Phycodnaviridae*, and *Iridoviridae*, which all infect eukaryotic hosts (9, 24, 33). This has led to the hypothesis that all these viruses belong to the same lineage, with a common ancestor existing before the separation of the three domains of life (5, 6).

Phage Bam35 is closely related to *Bacillus cereus* plasmid pBClin15. Bacilli harbor a large variety of plasmids, which include a linear species of ≈15 kb (2, 13, 15, 31, 34). Recently, the genome sequencing of *Bacillus cereus* ATCC 14579 revealed the sequence of a linear plasmid (pBClin15) of 15,100 bp (19). While studying Bam35, we matched putative genes of the pBClin15 plasmid in independent database searches for the Bam35c coat protein and ATPase. After submission of this note, the nucleotide sequence of a linear *B. thuringiensis* plasmid, pGIL01, which differs from the Bam35c sequence by only approximately 10 nucleotides was published (32).

To investigate the relationship of pBClin15 to Bam35c (14,935 bp), their genomes were compared. The organizations of the open reading frames (ORFs) of pBClin15 and those of Bam35c are highly similar (Fig. 1). Moreover, their sequences agree with 45 to 81% identity. The corresponding amino acids have 18 to 88% identity (Table 1). The gene identification of Bam35c (25) was based on the reasonably detailed understanding of the corresponding PRD1 genes (7, 8, 16). A similar annotation shows that pBClin15 is related to Bam35 and PRD1 (Table 1). Among the most conserved genes in pBClin15 are those assigned to viral capsid components. These include the major coat protein 15 (corresponding to PRD1 protein P3) and the unique vertex packaging proteins 12, 14, and 16 (proteins P9, P20, and P22, respectively, in PRD1). Interestingly, the most conserved protein corresponds to a LexA-type transcription regulator (17, 21) homologue found in Bam35 but absent in PRD1.

We then explored the relationship between pBClin15, Bam35c, and PRD1 by comparing their major coat proteins. First, the three sequences were aligned (Fig. 2A). A model was then made of the pBClin15 protein, based on an earlier threading of the corresponding Bam35c sequence onto the high-resolution structure of the PRD1 coat protein, P3 (9, 10; unpublished results) (Fig. 2B). The three proteins clearly have the same fold, although the sequence similarity between Bam35c and PRD1 is very low (12% identity). In contrast, the Bam35c and pBClin15 proteins are very similar (65% identity), with their differences scattered throughout the molecule (Fig. 2B). Of note, two regions in PRD1 P3 (the N terminus and I1B2 loop) that interact with the internal membrane (28, 29) are shorter in Bam35c and pBClin15. The N-terminal helix shows some conservation in key residues but lacks the flexible tip of PRD1 P3 (Fig. 2B).

Plasmid pBClin15 lacks inverted terminal repeats. Microbial extrachromosomal linear elements so far characterized are divided into two groups: those carrying covalently closed ends (hairpins) and those with covalently attached 5′-terminal proteins, similar to those in viral genomes (22, 26). Bacteriophage PRD1 contains inverted terminal repeats, has covalently linked proteins at its 5′ ends, and replicates using a protein-primed mechanism (3, 7, 11, 30). Bam35c also has inverted terminal

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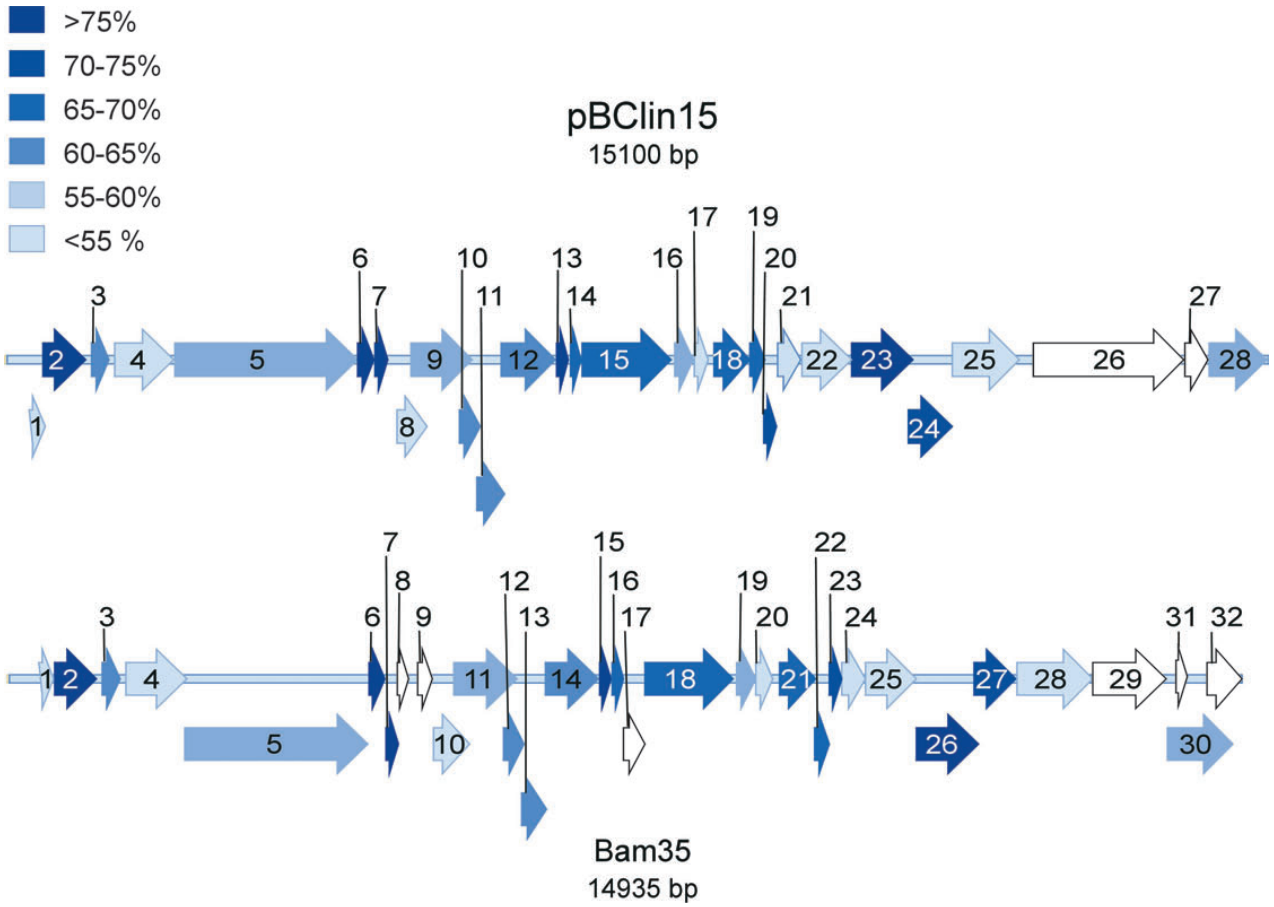

FIG. 1. A comparison of the pBClin15 and Bam35 genomes. Open reading frames are depicted by block arrows, shaded to show the level of DNA sequence identity with the corresponding gene in the other phage. Open reading frames with no counterpart are shown in white. The three levels of arrows reflect the different reading frames.

repeats and may have terminal proteins, as suggested by the finding that the migration of Bam35c DNA in agarose gels is dependent on protease treatment (25). The 5′ ends of the nearly identical pGIL01 DNA are also protected by terminal proteins: in addition to its similar protease-dependent gel migration, pGIL01 is only degraded by exonuclease III (a 3′-nuclease) but not by λ nuclease (a 5′-nuclease) (32). Comparison of Bam35c and pBClin15 genome termini revealed similar 5′ noncoding regions (over 70% identity at DNA level) of about equal length, but pBClin15 lacks the inverted terminal repeats.

Phage Bam35c can convert to a prophage. We investigated whether Bam35c can establish a carrier state, as proposed by Ackermann et al. (1). Lysogenic cell lines were obtained by picking microcolonies from the centers of plaques, as well as from confluently lysed plates of *B. thuringiensis* serovar *israelensis* HER1410 (obtained from the Félix d'Herelle Reference Center for Bacterial Viruses, Laval University, Quebec, Canada) infected with Bam35c. To eliminate remaining free phage particles, single-colony isolations were performed (a total of eight passages). Two of the cell lines obtained, named HER1410_L5 and HER1410_L7, were shown to contain Bam35c-specific sequences by PCR from single bacterial colonies (for method, see reference 20) with specific primers hybridizing to the ends of Bam35c genes 6 and 14 (GenBank accession no. AY257527) (Fig. 3A).

The isolated lysogenic cell lines released viruses into culture supernatants (typically 10^2 to 10^3 PFU/ml after 8 h of growth of cells in Luria-Bertani (27). The phage were shown to arise from Bam35c by PCR from single plaques (for method, see reference 18) with specific primers as described above (Fig. 3A). The virus-producing cell lines now carried ≈15-kbp DNA elements, the Bam35c prophage, that were not present in the original HER1410 (Fig. 3B).

Bam35-like prophage are common in bacilli. To check the distribution of similar prophages, we investigated several *B. thuringiensis* strains from the Bacillus Genetic Stock Center (Ohio State University, Columbus). Of seven strains tested, four released viruses into the culture supernatant that were detectable on the Bam35 host strain HER1410. One of these, *B. thuringiensis* serovar *israelensis* 4Q4 (WHO2013-9), was identified as carrying a Bam35c-related prophage by colony and plaque PCR as described above (Fig. 3A). As with the virus-producing cell lines isolated previously, 4Q4 contains a specific ≈15-kbp DNA element not found in HER1410 (Fig. 3B).

TABLE 1. Comparison of Bam35c genes with pBClin15 ORFs at the protein and DNA levels[a]

Bam35c Protein (no. of residues)	pBClin15 protein (no. of residues)	Location on pBClin15 (nt)	Identity (%)	Identity at DNA level (%)	Protein function[b]	PRD1 protein (no. of residues)
1 (58)	1 (63)	260–451	17.5	45.9		
2 (167)	2 (167)	432–935	84.5	81.3		
3 (74)	3 (71)	1010–1225	43.2	62.2		
4 (245)	4 (233)	1290–1991	31.0	50.0		
5 (735)	5 (729)	2004–4193	46.9	58.5	DNA polymerase	P1 (553)
6 (66)	6 (66)	4196–4396	87.9	79.1	Lex A-type repressor	
7 (50)	7 (49)	4412–4561	70.0	75.8		
8 (46)						
9 (57)						
10 (145)	8 (118)	4671–5027	36.3	52.8	Unique vertex	P6 (166)
11 (252)	9 (243)	4838–5569	36.0	56.5	Assembly	P10 (203)
12 (80)	10 (81)	5415–5660	44.4	60.2		
13 (102)	11 (106)	5629–5949	59.4	64.8		
14 (212)	12 (216)	5921–6571	62.5	63.6	Packaging ATPase	P9 (227)
15 (46)	13 (46)	6584–6724	78.3	78.0		
16 (46)	14 (45)	6739–6876	65.2	69.5	Unique vertex	P20 (42)
17 (84)						
18 (356)	15 (355)	6880–7947	65.2	66.4	Capsid protein	P3 (395)
19 (76)	16 (76)	7988–8218	42.9	58.7	Unique vertex	P22 (47)
20 (68)	17 (57)	8221–8394	32.4	52.7		
21 (143)	18 (140)	8468–8890	61.9	65.2		
22 (58)	19 (58)	8891–9067	67.2	69.5		
23 (48)	20 (48)	9064–9210	72.9	71.4		
24 (91)	21 (92)	9222–9500	35.9	53.0		
25 (207)	22 (197)	9513–10106	42.4	54.3	Infectivity	P11 (207)
26 (250)	23 (243)	10110–10841	76.8	75.0	Transglycosylase	P7 (265)
27 (170)	24 (173)	10789–11310	79.9	74.9		
28 (304)	25 (264)	11323–12117	20.2	48.3		
	26 (597)	12290–14083				
29 (293)						
	27 (95)	14096–14383				
30 (265)	28 (227)	14396–15079	57.2	59.5	Endolysin	
31 (40)						
32 (141)						

[a] Each ORF was compared with the other whole genome (Align X/ Vector NTI 7.0). The ORFs of pBClin15 and the corresponding genes of Bam35c and their proteins were then compared individually. The known biochemical and structural properties of related PRD1 proteins and proposed functions of Bam35 proteins are listed in the last two columns. The accession number for Bam35c is AY257527. The accession number for pBClin15 is NC_004721. The genome has been reannotated, and the ORF numbering does not correspond to that used in GenBank. Stop codons are included in the given nucleotide coordinates. Location numbering for pBClin15 refers to NC_004721.

[b] From reference 25.

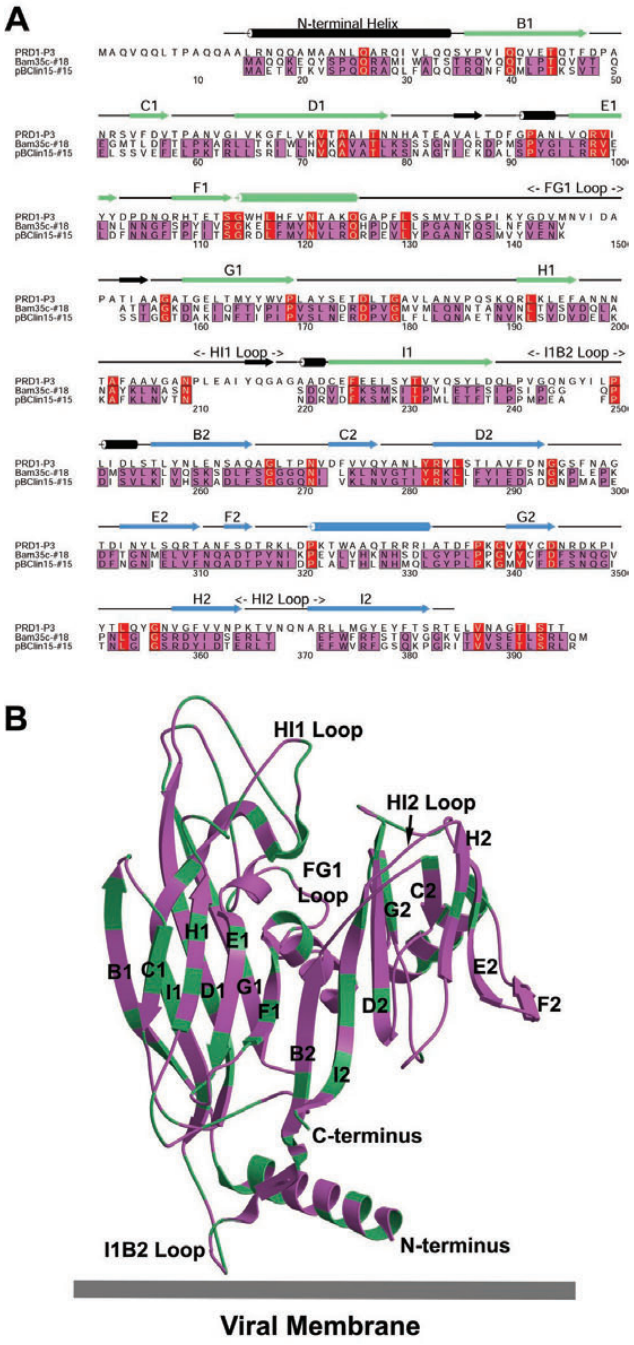

FIG. 2. Major capsid proteins. (A) A sequence alignment of the major coat protein, P3, of phage PRD1 with those predicted for Bam35c (protein 18) and pBClin15 (protein 15). The *Bacillus* phages and prophages show high identity (65%; purple), whereas PRD1 P3 is more distantly related (12%, red). The secondary structural elements for PRD1 P3, determined by X-ray crystallography, are shown above the alignment with α-helices as rods and β-strands as arrows. The two eight-stranded viral jelly rolls that define the structure are shown in green and blue (strands marked B1-I1 and B2-I2 [9]). Note that the deletions in the *Bacillus* phage/prophage relative to PRD1 occur in the loops connecting the strands of the jelly rolls. These affect the loops at the top of the molecule forming the viral surface (FG1, HI1, and HI2) and the I1B2 loop at the base (see below). (B) A model of the pBClin15 coat protein based on a threading of the corresponding Bam35c sequence onto the PRD1 P3 crystal structure with the alignment as a guide. The residues that are identical in the Bam35c and pBClin15 proteins are shown in purple, and the ones that differ are in green. Interactions with the membrane occur through residues in the N-terminal helix and I1B2 loop at the base of the molecule. Both features are shorter in Bam35c and pBClin15 than in PRD1.

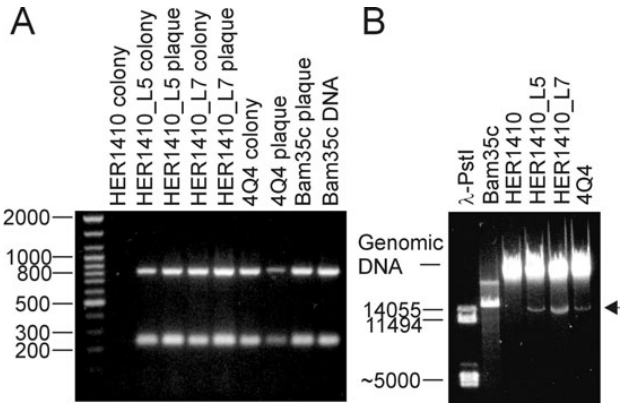

FIG. 3. Detection of Bam35-related genomes. (A) PCR amplification of Bam35-specific sequences from lysogenic cell lines and plaques with specific primers against Bam35c ORFs 6 and 14, producing PCR products of 235 bp and 810 bp, respectively. Lane 1 contains Generuler DNA ladder mix (MBI Fermentas). Lanes 2, 3, 5, and 7 contain PCR products obtained by colony PCR of host strain HER1410, lysogenic cell lines HER1410_L5 and HER1410_L7, and strain 4Q4, respectively. Lanes 4, 6, 8, and 9 show PCR products obtained by plaque PCR of cell lines HER1410_L5 and HER1410_L7, strain 4Q4, and a Bam35c plaque, respectively. Lane 10 contains the PCR products obtained from purified Bam35c DNA (control). (B) The lysogenic cell lines HER1410_L5 and HER1410_L7 and strain 4Q4 carry ≈15-kbp DNA molecules that cannot be found in the original Bam35 host strain HER1410. Lanes 1 and 2 contain purified *Pst*I-digested λ DNA and purified Bam35c DNA, respectively. Lanes 3 to 6 contain purified DNA (Wizard Genomic DNA purification kit; Promega) from host strain HER1410, lysogenic cell lines HER1410_L5 and HER1410_L7, and strain 4Q4, respectively. The arrowhead depicts the ≈15-kbp DNA element found in the lysogenic cell lines and strain 4Q4.

Is the Bam35 carrier state maintained by protein-primed replication? The *Bacillus anthracis* phage AP50 (23) is also related to Bam35 and PRD1 (4). We have shown here that *B. thuringiensis* strains carry Bam35-like prophages and that *B. cereus* plasmid pBClin15 is closely related to Bam35. Obviously, these three bacilli carry related phage/prophage systems. The likely mechanism by which the prophage state is maintained is intriguing. The Bam35c genome, analogously to PRD1, may contain terminal proteins (25). These proteins are used as primers for initiating replication, and protein-primed replication mechanisms occur in lytic bacteriophages, such as PRD1 and ϕ29, and also in adenovirus and linear plasmids (26). The idea that protein-primed replication can also operate in the carrier state is novel, as this mechanism has so far not been reported for prophages (12, 14). As pBClin15 does not contain inverted terminal repeats, it may be a degenerating prophage that cannot give rise to virus particles. These observations open interesting avenues for future research in the Bam35-like virus-plasmid system.

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In vitro DNA Packaging of PRD1: A Common Mechanism for Internal-membrane Viruses

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PRD1 is the type virus of the *Tectiviridae* family. Its linear double-stranded DNA genome has covalently attached terminal proteins and is surrounded by a membrane, which is further enclosed within an icosahedral protein capsid. Similar to tailed bacteriophages, PRD1 packages its DNA into a preformed procapsid. The PRD1 putative packaging ATPase P9 is a structural protein located at a unique vertex of the capsid. An *in vitro* system for packaging DNA into preformed empty procapsids was developed. The system uses cell extracts of overexpressed P9 protein and empty procapsids from a P9-deficient mutant virus infection and PRD1 DNA containing a LacZα-insert. The *in vitro* packaged virions produce distinctly blue plaques when plated on a suitable host. This is the first time that a viral genome is packaged *in vitro* into a membrane vesicle. Comparison of PRD1 P9 with putative packaging ATPase sequences from bacterial, archaeal and eukaryotic viruses revealed a new packaging ATPase-specific motif. Surprisingly the viruses having this packaging ATPase motif, and thus considered to be related, were the same as those recently grouped together using the coat protein fold and virion architecture. Our finding here strongly supports the idea that all these viruses infecting hosts in all domains of life had a common ancestor.

Keywords: DNA packaging ATPase; internal membrane-containing virus; evolution

Introduction

An essential step in virus assembly is the incorporation of viral nucleic acid into the virion. There are two basic mechanisms: some viruses assemble their protein shell and package their genome simultaneously in a co-assembly reaction, while others first produce empty procapsids into which the genome is specifically packaged. The translocation of nucleic acids into procapsids is an energy-requiring reaction propelled by a specific enzyme, the packaging NTPase (or terminase, if the replication product is concatemeric DNA). Bacteriophages are the best understood models for this latter mechanism, as *in vitro* packaging systems have been developed for several of them, as described below. In these viruses, packaging occurs through a ring-like portal structure at a unique vertex of the viral capsid. In tailed double-stranded (ds) DNA bacteriophages, this is also the site for tail attachment. Packaging into preformed particles may be used by several eukaryotic dsDNA viruses, such as the herpesviruses.[1] A similar packaging mechanism is also in use among RNA viruses, demonstrated by the *in vitro* packaging system for dsRNA bacteriophage phi6 and its relatives. In the phi6 system, single-stranded RNA is translocated into a preformed capsid where it is replicated to double-stranded form.[2–4]

In vitro packaging systems have been developed for tailed dsDNA bacteriophages using slightly different encapsidation mechanisms: (a) phages using terminases cutting and packaging the DNA in a headful manner, such as P22,[5,6] SPP1,[7] T4,[8,9] and T1;[10] (b) phages cutting their concatemeric DNA only at specific cut sites, such as lambda,[11] T3,[12] and T7;[13] and (c) phages having unit-length DNA genomes that are not cleaved during packaging, such as phi29.[14,15] Of these, the packaging of bacteriophage phi29 is probably the best characterised. phi29 has covalently linked terminal proteins in its linear genome,[16] and curiously, a procapsid-bound RNA molecule (pRNA, or

Abbreviations used: ds, double-stranded; pfu, plaque-forming units; cfu, colony-forming units.

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prohead RNA) playing an important role in DNA packaging.[17] No *in vitro* packaging system has yet been described for any membrane-containing DNA virus.

PRD1 is a tail-less icosahedral bacteriophage with a 15 kb linear dsDNA genome. It is the type virus of the *Tectiviridae* family and infects a broad range of Gram-negative bacteria.[18] There are five virus isolates (PR3, PR4, PR5, L17 and PR772) almost identical in sequence with PRD1 (A. M. Saren *et al.*, unpublished results).[19] In addition to these, the *Tectiviridae* include the Gram-positive bacteria-infecting bacteriophages AP50,[20] Bam35[21,22] and pGIL01.[23] Recently, a putative prophage or defective phage, pBClin15, which is similar to Bam35 in sequence and gene organisation, was also discovered.[24,25] PRD1 and the other tectiviruses differ from tailed phages in that they have an internal lipid membrane underneath their protein capsid, surrounding the genome. The membrane plays an active role in DNA delivery to the host cell by forming a tubular structure used for injecting the DNA into the host cytoplasm.[26–28] The ~650 Å diameter capsid of PRD1 is formed of 240 copies of major capsid protein P3 trimers. The spike complexes at the 5-fold vertices are formed of penton protein P31, spike protein P5 and receptor binding protein P2.[29–31] The recent crystal structure of PRD1 reveals the details of the capsid architecture[32] and gives insights into the arrangement of the internal membrane.[33]

PRD1 has a unique vertex, which extends to the internal membrane *via* two small integral membrane proteins, P20 and P22. Minor capsid protein P6 attaches to the virion *via* these proteins and is, in turn, necessary for the attachment of the putative packaging ATPase P9 to the virion.[34,35] Unlike many other packaging ATPases, P9 is a structural protein and stays associated with the mature virion.[36] Nothing is known of a possible portal protein or its structure. In viruses where DNA packaging has been studied in more detail, packaging is accompanied by expansion of the procapsid to form the mature capsid structure.[37] Such a capsid expansion does not occur in PRD1. Instead, when empty and filled particles were compared using cryo-electron microscopy and image reconstruction, the internal membrane was slightly expanded in particles filled with DNA, leading to more intimate contacts between the membrane and the major capsid protein P3 shell.[33,38,39]

The structural and architectural features of PRD1 were compared with those of adenovirus, leading to the discovery of a number of similarities. PRD1 and human adenovirus share the same major coat protein fold, a trimeric double β-barrel.[40] Both viruses have the same pseudo $T=25$ capsid arrangement[38,41] not seen in any other virus. PRD1 and adenovirus also share many other common features, such as a common vertex organisation, the presence of inverted terminal repeats and 5′-terminal proteins at the ends of their linear genomes, and a protein-primed DNA replication mechanism.[42–44] Based on these similarities, PRD1 and adenovirus have been suggested to originate from a common ancestor.[40,45] However, the absence of a membrane in adenovirus is an obvious difference.

The PRD1/adenovirus type capsid can be scaled up to accommodate larger genomes, e.g. the same coat protein fold has recently been found in the algae-infecting *Paramecium bursaria* chlorella virus (PBCV-1), belonging to the *Phycodnaviridae* family, and also having an internal membrane ($T=169$, capsid diameter 1900 Å).[46,47] The trimeric double β-barrel coat protein fold seen in PRD1, adenovirus and PBCV-1 has, based on modelling, been proposed to exist in several other viruses, such as Bam35, Chilo iridescent virus (CIV, *Iridoviridae*), African swine fever virus (ASFV-1, *Asfarviridae*) and Mimivirus.[21,48] These viruses are all icosahedral dsDNA viruses, of which Bam35, CIV and ASFV-1 are known to contain an internal membrane.[22,49–53] The conservation of a core set of genes in viruses of the *Phycodnaviridae*, *Asfarviridae* and *Iridoviridae* families, and recently also Mimivirus, has led to proposals of their relatedness.[54,55] This group was further expanded by the suggestion that the internal membrane-containing viruses of the *Ascoviridae* family originate from an iridoviral ancestor.[56] Iyer *et al.*[54] further proposed that members of the *Poxviridae* should also be included in this group. Recently, an archaeal virus, Sulfolobus turreted icosahedral virus (STIV), was shown to share the same architectural principle with PRD1 and adenovirus. STIV may also contain an internal membrane.[57] The structural and sequence comparisons of these viruses have led to the proposal that the above-mentioned viruses infecting hosts in all three domains of life belong to the same lineage originating from a common ancestor.[48,58,59]

We have developed an *in vitro* system for the analysis of DNA packaging in bacteriophage PRD1 and have experimentally shown that the PRD1 protein P9 is needed for the packaging. Database searches with PRD1 P9 showed similarities to putative viral packaging ATPases of members in the proposed viral lineage but not to known packaging ATPases of other types of viruses, such as the tailed dsDNA bacteriophages. Regions of conserved amino acid residues in the putative PRD1 packaging ATPase P9 and the other putative ATPases of membrane-containing viruses were identified. In order to establish their significance, conserved amino acid residues in P9 were mutated and the mutant proteins were tested for their packaging ability in both *in vivo* and *in vitro* systems.

Results

Development of an *in vitro* DNA packaging system for bacteriophage PRD1

Upon packaging, the linear genomic DNA molecule of PRD1 traverses both the external protein

capsid and the internal membrane vesicle at the specific portal vertex. In the packaging reaction purified recombinant PRD1-1 DNA containing a *lacZ*α-insert, extracts containing recombinant P9 protein, and empty particles obtained from P9-deficient Sus1 mutant infection were mixed. The key to establishing the *in vitro* packaging system for PRD1 was distinguishing the packaged exogenously supplied DNA molecules from those endogenously present in the system. Successful packaging of exogenous DNA resulted in the formation of blue plaques scored by plating on a host providing the LacZω fragment (*Escherichia coli* DH5α (pJB15)). The packaging mixture was treated with DNase before plating on the host to remove any unpackaged DNA. Consequently, the blue plaques were formed by packaged DNA only and thus empty viral particles produced using the *sus1* mutant are true procapsids, which can be productively packaged. In the absence of exogenous PRD1-1 DNA or in the case of DNase digestion directly after addition of the PRD1-1 DNA to the reaction, no blue plaques were seen. A constant but very low background of white plaques was seen in all reactions. These white plaques arise from a background of packaged virions from the Sus1 particle extract.

The energy for DNA translocation was specifically provided by adenine nucleoside triphosphates with no signal (0 blue plaques/100 µl of undiluted packaging reaction mix) obtained with the three other NTPs (GTP, CTP and UTP). Interestingly, the reaction was less stringent with respect to the sugar moiety, as both dATP and ddATP supported the packaging reaction, albeit with a somewhat lower efficiency than ATP (Figure 1(a)). The amount of ATP used was 2.5 mM. This was sufficient to support the packaging reaction, as addition of more ATP (up to 10 mM) either at the beginning or during the reaction (at 30 minutes) did not increase the number of plaques formed. Lesser amounts of ATP produced significantly lower yields of packaged virions. The reaction was also, to a large extent, dependent on the addition of external Mg^{2+} but not Ca^{2+} as indicated in Figure 1(b) (compare "no $MgCl_2$", "no $CaCl_2$" and "2.5 mM EDTA" with "optimised", which represents the optimised reaction). Polyethylene glycol 8000 (PEG) enhanced the packaging considerably (Figure 1(b)). The packaging reaction had a reasonably broad pH optimum (pH 6–8, not shown) but omission of the buffer had an adverse effect (Figure 1(b)). Several different buffering substances (Tris (pH 7.2 and 8.0), Bis-Tris (pH 6.5), piperazine (pH 5.5), sodium phosphate (pH 7.2), potassium phosphate (pH 7.2) and sodium acetate (pH 7.2)) were preliminarily tested, and all buffers that were used at near-neutral pH were found to enhance packaging almost as well. However, Tris (pH 7.2) proved to perform best and was chosen for the standard conditions. The volume of extracts, temperature and reaction time were optimised (Figure 1(c)–(f)) and consequently, standard reaction conditions (37 °C, 45 minutes, 15 µl of P9 and

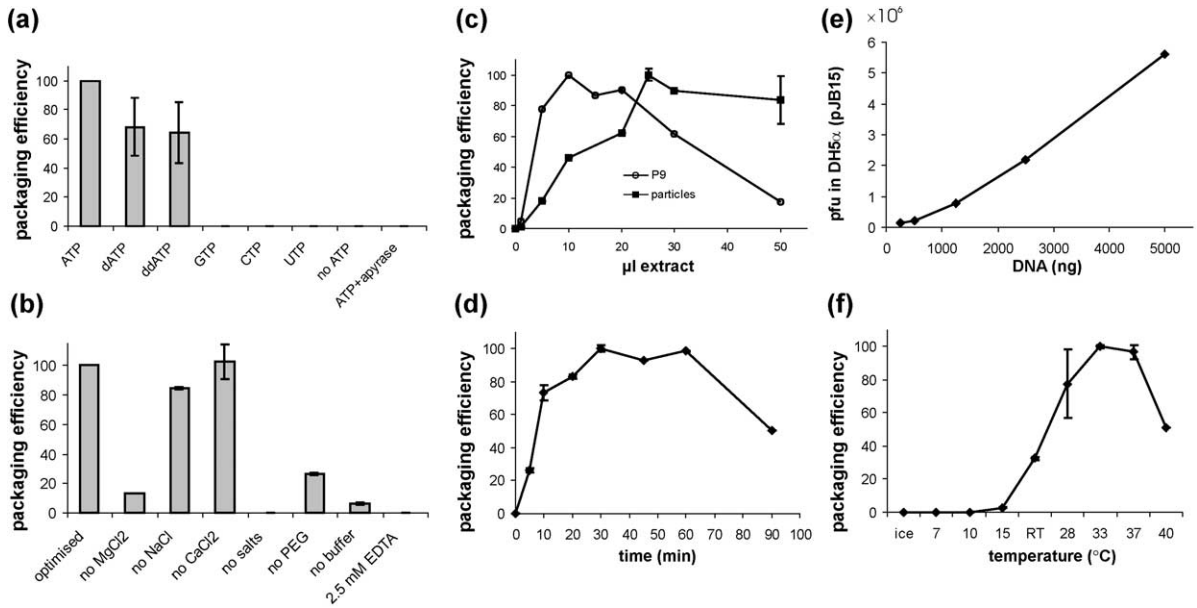

Figure 1. Optimisation of the PRD1 *in vitro* packaging system and the effect of different nucleotides and ionic conditions on the *in vitro* DNA packaging efficiency. (a) Nucleotide requirements of PRD1 DNA packaging, (b) effects of salts, PEG and buffering on packaging efficiency, (c) optimisation of amounts of ATPase and procapsid extracts, (d) effect of incubation time, (e) amount of DNA and (f) temperature on packaging efficiency. In (a)–(d) and (f), packaging efficiency is shown as the relative packaging efficiency (%); phage yield (pfu/ml) obtained in each reaction is compared to 100% packaging efficiency ($\sim 10^5$ pfu/ml) obtained with the optimised standard reaction with 250 ng of DNA.

25 μl of particle extract) were chosen. As shown in Figure 1(e), it was not possible to saturate the DNA amount added to the reaction, probably due to the strong adhesive property of the genome terminal protein interfering with the DNA purification.[42,60]

Plating of the reaction mixture on *E. coli* DH5α (pJB15) resulted maximally (when 5000 ng of DNA was used) in 6×10^6 plaque-forming units (pfu)/ml (blue plaques) of the packaging reaction. A background of $\sim1\times10^3$ pfu/ml (white plaques) arising from packaged virions in the Sus1 particle extract could be observed when either P9 or DNA was omitted from the reaction. Approximately 250 ng of DNA, which produced around 10^5 packaged virions when assayed on *E. coli* DH5α, was included in the standard reaction; however, the efficiency of different DNA batches varied somewhat. The reaction was insensitive to the addition of RNases (RNaseA, RNaseI or RNaseIII, data not shown), suggesting that there are no RNA molecules involved in PRD1 packaging, as in bacteriophage phi29.

When the standard PRD1 host *Salmonella enterica* DS88 was used, approximately 2.4×10^8 pfu/ml of extract were obtained (background of white plaques $\sim3\times10^4$ pfu/ml) with 5000 ng of DNA (this is equivalent to 6×10^6 pfu/ml in *E. coli* DH5α). The plaques formed on DS88 were also tested on *E. coli* DH5α (pJB15) and were confirmed to yield blue plaques. The difference between the hosts reflects the poor receptor expression in *E. coli*.[61] The standard reaction contained approximately 3 nmol of P9/ml (calculated as a monomer) and 6×10^{11} procapsids/ml, corresponding to 1 pmol/ml (calculated on the basis that each prematurely lysed infected cell releases 50 procapsids). Up to 5 μg (approximately 0.5 pmol) of DNA was used to obtain the highest titres. The packaging efficiency of a representative DNA preparation (assayed by plating on DH5α) is shown in Figure 1(e).

Comparison of tectiviral putative packaging ATPase sequences reveals a specific consensus motif

The amino acid sequence of P9 was aligned with the corresponding putative ATPase sequences of Bam35 and pBClin15, the PRD1-related phages/plasmids replicating in Gram-positive hosts (Figure 2). These putative ATPases are of a similar length to P9, but share only limited sequence similarity with it. Three regions comprising several similar or identical residues were observed: two of the conserved regions were easily identified as the classical Walker A (⟨hydrophobic stretch⟩[GA]XXGXGK[ST]) and B (⟨hydrophobic stretch⟩D[ED]) ATPase motifs.[62,63] The third region, comprising several identical amino acid residues (starting ALXTGRSK and ending DR), and located downstream of the Walker B motif, could not be identified as any previously described ATPase motif, and was assigned as the P9-specific motif. Several other short regions of identical amino acids (e.g. IY-PI) were detected preceding the Walker B motif (Figure 2).

Database searches for P9-like proteins

Iterative database searches with the putative PRD1 ATPase P9 amino acid sequence as the seed were performed with PSI-Blast,[64] both using the whole available non-redundant protein sequence database (National Center for Biotechnology Information, NIH) and by restricting the search to viral sequences. Distribution of identical or similar amino acids was required to be over the whole length of the sequences. Only proteins of similar length to PRD1 P9 were accepted, so that proteins with long additional N or C-terminal regions were not included.

The sequences retrieved were almost exclusively from dsDNA viruses with an internal membrane.

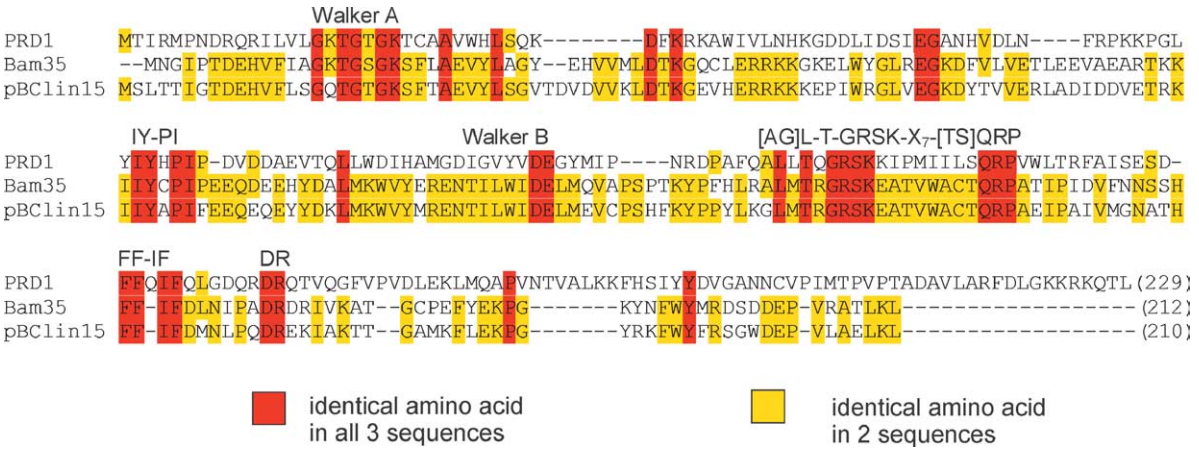

Figure 2. Comparison of putative tectiviral packaging ATPases. Putative ATPase sequences of Bam35 and pBClin15 were aligned with PRD1 P9. Amino acid residues identical in all three sequences are depicted in red, and amino acid residues identical in only two of the sequences are depicted in yellow. Accession numbers: PRD1 P9 (P27381), Bam35c orf 14 (NP_943760), pBClin15 orf12 (NP_829897).

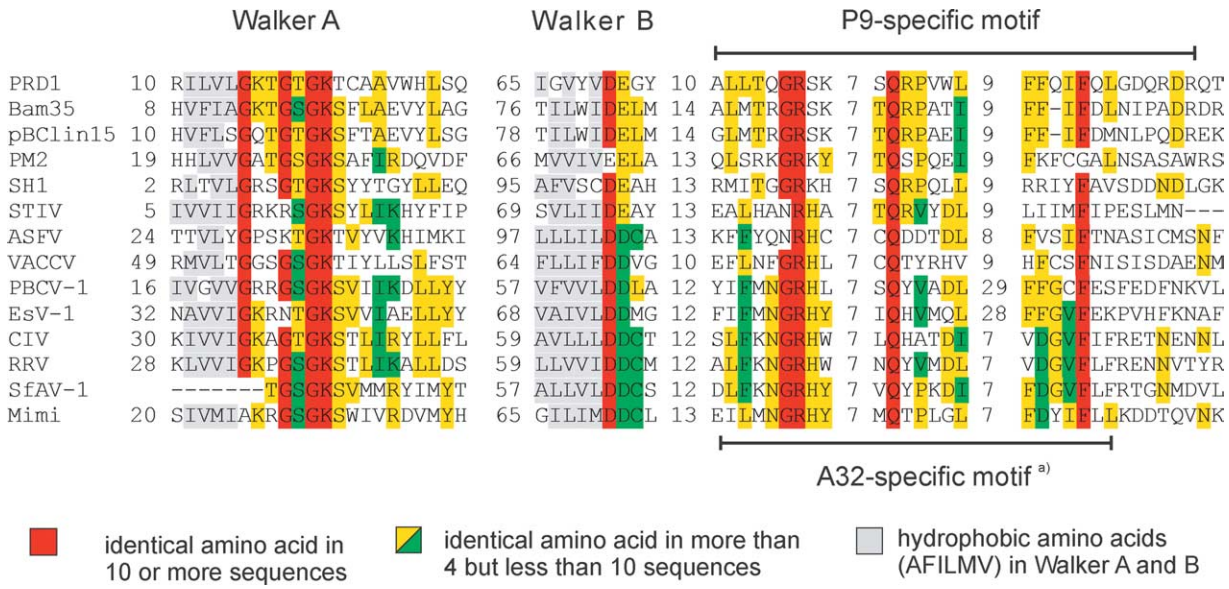

Figure 3. Comparison of putative ATPase sequences from membrane-containing dsDNA viruses of bacterial, archaeal and eukaryotic origin. PRD1 P9 and putative ATPase sequences from bacterial (Bam35, pBClin15), archaeal (SH1, STIV) and eukaryotic (ASFV, PBCV-1, EsV-1, CIV, RRV, VACCV, SfAV-1 and Mimivirus) viruses are aligned (the acronyms are defined below). Sequences were chosen either on the basis of previously proposed evolutionary relationships of the viruses, based on structural and genetic comparisons, or sequence similarity to PRD1 P9, as detected by PSI-Blast. Amino acid residues identical in ten or more of the sequences are depicted in red, and residues identical in more than three but less than ten sequences are depicted in yellow and green (which is used in the case of two groups of more than three to nine identical amino acid residues). Putative ATPases and their accession numbers: PRD1 P9 (P27381), Bam35c orf 14 (NP_943760), pBClin15 orf12 (NP_829897), SH1 = archaeal virus SH1 orf 17 (AY950802), STIV = Sulfolobus turreted icosahedral virus B164 (AAS89100), ASFV = African swine fever virus pB354L (NP_042772), PBCV-1 = *Paramecium bursaria* chlorella virus 1 A392R (NP_048749), ESV-1 = *Ectocarpus siliculosis* virus 1 orf 26 (NP_077511), CIV = Chilo iridescent virus ATPase 075L (T03048), SfAV-1 = *Spodoptera frugiperda* ascovirus 1 ATPase 3 (CAC84470), RRV = *Regina ranavirus* ATPase (YP_003858), VACCV = vaccinia virus A32 (P21055), Mimi = Mimivirus VV A32 virion packaging ATPase (AAV50705).

In the first PSI-Blast search, only other tectiviral ATPase sequences were retrieved. In the next search round, including now the putative Bam35 and pBClin15 ATPase sequences, no other putative bacteriophage ATPases were found, but instead, several Ranavirus (viruses belonging to the *Iridoviridae* family) sequences were identified. In the next iteration, phycodnaviral, ascoviral and other iridoviral sequences could be retrieved, in addition to a putative ATPase sequence from Mimivirus, followed by poxvirus sequences. The archaeal virus STIV B164 protein and the P9 protein of PM2, another membrane-containing dsDNA bacteriophage,[65,66] were also retrieved. When the putative ATPase of Bam35 was used as the seed, the results were similar: first only tectiviral sequences were retrieved, then the putative ATPase of bacteriophage PM2, followed again by sequences from the ranaviruses. The sequence of the putative ATPase of SH1,[67] an archaeal dsDNA virus with an internal membrane, was also included in the analysis. When a database search was done with SH1 putative ATPase, the first viral sequence identified was PRD1 P9.

No packaging ATPases of tailed phages (such as phi29, lambda, P22 or T4) or eukaryotic viruses belonging to other than the above-mentioned families (such as herpesvirus) could be retrieved with the PRD1 or Bam35 sequences with any parameters used. And *vice versa*, the PRD1 putative ATPase could not be identified from the databases using phi29, T4 or herpesvirus packaging ATPase/terminase sequences.

Multiple alignment of bacterial, archaeal and eukaryotic virus putative ATPase sequences

The tectiviral ATPase sequences were aligned with putative viral ATPase sequences retrieved with PSI-Blast. Additionally, putative ATPase sequences from other eukaryotic and archaeal viruses, which may be structurally related to PRD1 such as ASFV and halovirus SH1, were included in the alignment. For the comparison, either the type virus of each family or genus was chosen, or in some cases, the member of the family first picked up in the iterative database search with PRD1 P9.

All the compared ATPases were of approximately similar length, and Walker A and B motifs could be detected at similar positions as in the tectiviral sequences (Figure 3). The Walker B motif ends -DD in the eukaryotic viruses and -DE in the bacteriophages and archaeal viruses, the exception being

the -EE in the putative PM2 ATPase. The P9-specific motif is similar in sequence and position to the A32 motif identified earlier in Vaccinia virus protein A32 and putative ATPases of certain other eukaryotic viruses[54,68] (see Figure 3). The function of these putative ATPases in eukaryotic viruses has not yet been experimentally verified, except for poxviruses, where the A32 protein of Vaccinia virus has been shown to play a role in genome encapsidation.[69] A similar motif was also found in the archaeal virus sequences STIV protein B164 and the putative SH1 ATPase (Figure 3). With non-stringent parameters, adenovirus putative ATPase IVa2 could also be retrieved after several PSI-Blast rounds with PRD1 P9 as the seed. Taking into account the structural similarities between PRD1 and adenovirus, IVa2 was included in the preliminary analysis despite its larger size. However, clear similarity to the P9 or A32 specific motifs could not be seen in IVa2, and it was not included in the final alignment.

Probing the P9 conserved motifs with targeted mutagenesis

Single amino acid changes were introduced to the conserved residues of the three motifs in PRD1 P9. The resulting mutant constructions were tested by an *in vivo* complementation assay using a P9-deficient PRD1 mutant. A subset of the mutants was further tested in the *in vitro* packaging system. All the constructions were checked for production and solubility of the recombinant altered P9 protein. The solubility of the altered P9 proteins suggests that these proteins were properly folded. Results of the complementation assay and the *in vitro* packaging analyses of the mutants are shown in Table 1.

The Walker A motif of PRD1 contains two GKT triplets, of which the latter is more conserved when compared with other viral ATPases (Figure 3). To identify the critical lysine residue proposed to interact with the β and γ-phosphate groups of ATP,[70] both lysine residues were mutated to alanine and arginine (K17A, K22A, K17R, K22R). The K17R mutation had no effect whereas K22R abolished complementation, suggesting that K22 is the critical lysine residue (Table 1). P9 proteins with mutations in the Walker B motif (D103, E104) did not show any complementation activity, as expected. Mutations in the amino acid residues conserved in both the P9 and A32 specific motifs (R123, Q134) were deleterious to complementation. The effects of mutating amino acids in the P9-specific motif conserved only in the tectiviruses were either not as severe (T120), or the mutations had no detectable effects (K125A, R135) (Table 1). Mutating the conserved tyrosine between the Walker A and B motifs (Y74) to phenylalanine had no effect on complementation. Three and seven amino acid residues were deleted from the C terminus of P9, since it has been shown in some phages that the C-terminal parts of the terminase large subunits are important in prohead binding.[71,72] Both truncations resulted in loss of P9 complementation activity.

Table 1. Analysis of PRD1 P9 mutants by *in vivo* complementation analysis and *in vitro* packaging assay

Motif	aa change	Description	Complementation[a]	*In vitro* packaging[b]
	Negative control		-	-
	Positive control		+++	+++
Walker A	K17R	GKTGTGKT	+++	
	K17A	GKTGTGKT	-	
	K17Q	GKTGTGKT	-/+	-
	K22R	GKTGTGKT	-	
	K22A	GKTGTGKT	-	-
YIY	Y72A	YIY	-/+	-
	Y74A	YIY	-/+	
	Y74F	YIY	+++	+++
Walker B	D103A	GVYVDE	-/+	
	D103E	GVYVDE	-/+	
	E104Q	GVYVDE	-/+	
	E104D	GVYVDE	-	
packaging ATPase motif	T120A	ALLTGRSK	++	-
	R123A	ALLTGRSK	-	
	R123K	ALLTGRSK	-	
	K125A	ALLTGRSK	+++	+++
	Q134A	SQRP	-	
	Q134E	SQRP	-/+	
	R135K	SQRP	+++	-
	R135A	SQRP	-/+	
	F149V	FFQIF	-/+	
	F150A	FFQIF	-/+	
	F153A	FFQIF	-/+	
	P208R[c]	as in phage PR3	-/+	-/++
C-terminal deletions	ΔC3	C-term. 3 aa deletion	+/-	
	ΔC7	C-term. 7 aa deletion	+/-	

[a] The titre of *sus1* was determined on a host bacterium carrying the mutant gene *IX* constructions. The levels of titres (pfu/ml) were: (−) $<10^5$ (level of vector control); (+/−) 10^5-10^7; (+) $10^7-1\times10^9$; (++) 10^9-10^{10}; (+++) $>10^{10}$, level of positive control (wild-type P9).

[b] The packaging efficiency was determined by the *in vitro* DNA packaging assay. (−) No packaging, (++) 1–10% of wild-type efficiency, (+++) wild-type level.

[c] 10% of wild-type efficiency was achieved when a greater amount of mutant ATPase extract was used than for wild-type P9 in the *in vitro* packaging system. Greater amounts of IPTG had no effect on the *in vivo* complementation efficiency.

To confirm results obtained by the complementation assay, a subset of the mutants was tested in the *in vitro* packaging assay. The genes coding for the mutant proteins were transferred into the pMG60 expression vector, expressed, and the solubility of the proteins was analysed. Only soluble proteins expressed at wild-type (wt) level were included in the packaging assay. In the packaging assay, the tested mutants behaved as in the complementation assay, except for R135K (SQRP), which gave wild-type titres in the *in vivo* complementation assay but failed to package in the more stringent *in vitro*

packaging assay. Also T120A (ALL*T*GRSK), which had lowered but detectable activity in the complementation assay, did not package in the *in vitro* assay (Table 1).

The closely related PRD1 isolate PR3 releases only approximately 20% packaged particles upon lysis (the total amount of particles being equivalent to PRD1) whereas PRD1 yields approximately 80% filled particles, the rest being empty.[73] Comparing the genomic sequences of PRD1, PR3 and other close isolates (A. M. Saren *et al.*, unpublished results), the only unique difference revealed between the P9 proteins of PRD1 and PR3 was P208R. When this change was introduced into PRD1 P9, no complementation was observed *in vivo* but the packaging reaction with this recombinant P9 molecule produced plaques, provided that an increased amount (30 μl) of the extract was used. However, the efficiency of the packaging reaction with the P208R mutant P9 was ~10% of that obtained when wt P9 (15 μl) protein was used.

Discussion

We have here developed the first *in vitro* packaging system for analysing the DNA packaging mechanism of an internal membrane-containing virus, PRD1. It was shown previously that empty PRD1 wild-type particles are precursors of packaged ones,[36] and that these putative procapsids contain the viral internal membrane and all other packaging vertex proteins except P9.[35] It has also been shown that the putative packaging ATPase P9 is a structural component of mature virions.[36] Here, we show that empty particles from a P9$^-$ mutant (*Sus1*) infection will package the PRD1 genome in the presence of recombinant P9 protein. These mutant particles are produced in large amounts during infection, are homogeneous and contain all virion proteins except protein P9 as assessed by SDS-PAGE and Western blotting with all available antibodies to PRD1 structural proteins.

We propose a model for PRD1 DNA packaging where the specific vertex, consisting of at least the minor capsid protein P6 and small integral membrane proteins P20 and P22, the linear DNA-terminal protein complex and the putative P9 ATPase form a ternary complex that translocates the DNA into the membrane vesicle, which resides inside the capsid against an increasing pressure gradient. In contrast to the tailed phage systems, the PRD1 packaging ATPase remains a part of the portal complex, probably acting also as the cap. This is supported by the notion that P9 is always absent from wild-type empty procapsids and mutant particles, where any other of the packaging vertex proteins (P6, P20, P22) are missing, and is thus probably the last structural protein to be added to the virion upon assembly.[35]

The limiting factor in our *in vitro* packaging system is the genome. The number of procapsids used per 1 ml of reaction is approximately 6×10^{11} and that of genomes maximally 3×10^{11}. We assume, based on sequence similarity to certain hexameric ATPases[74] and the amount of P9 in the PR4 virion,[75] that P9 could act as a multimer, but even in such a case there is a vast excess of P9 in the reaction. Using the DS88 data for packaging, it can be calculated that up to 0.08% of genomes and 0.04% of the procapsids were productively packaged, yielding infective virions. We have observed previously that the PRD1 genome terminal protein is very adhesive, making the isolation of active DNA challenging. We assume that this causes the difference observed between separate DNA batches. The titres obtained with our PRD1 packaging system using 5000 ng of DNA (2.4×10^8 pfu/ml of packaging reaction, or 4.8×10^7 pfu/μg DNA) are comparable to those obtained with the *in vitro* packaging systems for phages SPP1 and phi29, for which titres of 1×10^7 and 6×10^7 pfu/ml of packaging reaction have been reported, respectively.[7,76] The commercial packaging kits available for bacteriophage lambda advertise titres ranging from 10^8 (Packagene® Lambda DNA packaging system, Promega) to 10^9 pfu/μg DNA (MaxPlax™ Lambda packaging extracts kit, Epicentre).

Sequence analysis of bacteriophage PRD1 P9 revealed, in addition to the classical Walker A and B ATPase motifs, a third conserved motif, which is not seen in packaging ATPases of other types of dsDNA viruses. The *in vitro* packaging system, together with the possibility of using a genetic complementation assay, allowed a more detailed analysis of PRD1 DNA packaging. The conserved regions of PRD1 P9 identified by sequence analysis were shown by the complementation and *in vitro* packaging assays to be essential. As all the viruses carrying the third motif (the P9/A32-specific motif) contain an internal membrane, we suggest that this region is a membrane-virus-specific packaging ATPase motif, identifiable in viruses infecting hosts in all three domains of life. In all the compared ATPases, the packaging ATPase motif (P9/A32) is composed of an arginine residue, usually preceded by a glycine residue, followed by a strictly conserved glutamine residue 11 amino acid residues downstream, and a conserved phenylalanine residue 17–19 positions further downstream (see Figure 3).

During the preparation of this article, a sequence analysis study was published grouping these (putative) viral ATPases as a clade of a new FtsK-HerA superfamily of ATPases.[74] The conserved glutamine residue and the preceding highly conserved arginine residue found in the putative viral ATPases were proposed to be a distinguishing factor for the whole superfamily when compared with other P-loop ATPases. The arginine residue was suggested to be analogous to the arginine finger of the AAA+ superfamily and the glutamine residue to be analogous to motifs playing a role in sensing the triphosphate moiety of the bound nucleotide and triggering ATP hydrolysis in the

AAA+ ATPases and SFI and SFII helicases. The putative dsDNA virus ATPases are positioned close to FtsK/SpoIIIE-type cellular proteins,[74] which are involved in transport of DNA across membranes or through septa, during cell division and sporulation, respectively.[77,78] The FtsK/SpoIIIE-type proteins have been suggested to act as multimeric (probably hexameric) DNA pumps.[79,80]

The lack of the third packaging ATPase motif presented here in the adenoviral packaging-related putative ATPase IVa2,[81] despite the many structural similarities between PRD1 and adenovirus, is interesting. Iyer *et al*.[74] place IVa2 separate both from the putative ATPases of PRD1 and the other membrane-containing dsDNA viruses analysed here, and from the terminases of tailed phages. This reflects our analysis, where IVa2 clearly differs from the tectivirus and other membrane virus ATPases, both due to the lack of the P9 or A32-specific motif and the presence of a large additional N-terminal domain, but still seems to have some similarity to P9, as analysed by PSI-Blast.

The bacteriophage T4 terminase large subunit gp17 has been suggested to contain features typical to the DEXD/H box helicases.[82] In the ATPases analysed in this study, the SH1 ATPase actually does have a DEAH-box signature in the Walker B motif, but no other helicase-specific motifs[83] could be observed in any of the ATPases described here. This, together with the fact that PSI-Blast searches with PRD1 P9 as the seed failed to retrieve ATPases (terminases) of tailed phages or herpesviruses, further suggests that the PRD1 P9-related ATPases belong to their own distinct group.

The similarities between the putative packaging ATPases of the membrane-containing eukaryotic, archaeal and bacterial viruses suggest a similar mechanism for the packaging of their dsDNA. The presence of a similar ATPase sequence in viruses infecting hosts in all domains of life suggests that these viruses may have had a common ancestor carrying this feature that existed before the separation of the current domains of life. It is of considerable significance that this very same conclusion has been drawn from comparisons of the viral coat protein architectures of these viruses.[40,48,58,59] The only exception is adenovirus, where the coat protein fold and virion architecture are equivalent to that of the other members of this virus lineage, but the ATPase is more remote. We propose that adenovirus originally had a membrane but lost it, and consequently adjusted its packaging system. Support for this comes from the observation that, despite the absence of the P9/A32 motif, the IVa2 ATPase is most similar to the PRD1 type of ATPases and not to any other known viral ATPase. The observation that the virion architectural principle, coat protein fold and packaging ATPase sequence are conserved within this virus lineage extends the idea of a viral "self" (a set of genetic determinants that encode virion structures that are functionally optimised and evolve slowly[58,59]) to include the packaging system.

Materials and Methods

Bacteria and phages

Cells were grown at 37 °C in Luria–Bertani (LB) medium[84] and when appropriate, chloramphenicol (25 μg/ml) or ampicillin (100 μg/ml) was added. PRD1-1,[31] with a *lacZα* fragment inserted into a unique PacI restriction enzyme site (nt 6307) in its genome, was propagated on *S. enterica* serovar Typhimurium LT2 DS88.[85] The P9 defective mutant *sus1* was propagated on suppressor strain PSA carrying the PRD1 receptor-encoding plasmid pLM2.[86]

For production of PRD1-1 virus particles, DS88 cells were infected at a multiplicity of infection (MOI) of approximately 8. After lysis of the cells, phage particles were purified by PEG/NaCl precipitation and 5%–20% rate-zonal sucrose gradient centrifugation, as described.[85]

DNA techniques

Plasmids used in this study are listed in Table 2. DNA manipulations were done using standard molecular biology techniques.[84] The pMG60 vector was constructed from pMS470Δ8.[87] The linker was changed to a more versatile one with multiple cloning sites. For this, the region between SacI and BamHI in pMS470Δ8 was removed and replaced with a synthetic linker containing NdeI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI and SphI recognition sites. The primase (*pri*) gene, unnecessary for this application, was further deleted from the vector by digestion with NdeI and SphI, Klenow polymerase treatment and ligation of the plasmid back to a circular molecule.

Gene *IX* coding for P9 was amplified, without its own ribosome binding site (RBS), by PCR using specific primers and inserted between the NdeI and HindIII restriction enzyme sites of plasmid pMG60. The resulting construct was named pNS96 and transformed into *E. coli* HMS174 cells[88] for protein expression.

Mutagenesis of gene *IX* was performed by PCR using the Quick Change® site-directed mutagenesis method (Stratagene) with specific, complementary primers containing altered nucleotides to produce the desired amino acid change and creating or destroying a suitable restriction enzyme site for screening. Plasmid pNS1 (gene *IX* cloned into pSU18) was used as a template and native *Pfu* DNA polymerase (Stratagene) as the enzyme. The constructs were sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems; at the DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, University of Helsinki). Constructs were transformed into HMS174 (pLM2) cells.

Complementation analysis

To verify that the host strains carrying constructs with mutations in gene *IX* were producing soluble P9 protein, the cells were grown at 37 °C to a cell density of 1×10^7 colony-forming units (cfu)/ml, induced with 1 mM IPTG, and grown overnight, collected, disrupted by sonication and the soluble and insoluble fractions were separated by centrifugation (Eppendorf microcentrifuge, 10,000 rpm, five minutes, 4 °C). Samples of the cultures were also

Table 2. Plasmids used in this study

Plasmid	Description[a]		Reference
pSU18	Cloning vector, p15A replicon, Plac, Cm^R		93
pMG60	Expression vector, ColE1 replicon, Ptac, Ap^R, T7 RBS		This study
pNS1	pSU18+PRD1 gene *IX* (nt 7624–8320)		36
pNS901	pNS1, altered P9	(K22R)	This study
pNS902	pNS1, altered P9	(K22A)	This study
pNS903	pNS1, altered P9	(Y74A)	This study
pNS904	pNS1, altered P9	(D103A)	This study
pNS905	pNS1, altered P9	(E104Q)	This study
pNS906	pNS1, altered P9	(T120A)	This study
pNS907	pNS1, altered P9	(R123A)	This study
pNS908	pNS1, altered P9	(R123K)	This study
pNS909	pNS1, altered P9	(Q134A)	This study
pNS910	pNS1, altered P9	(R135K)	This study
pNS911	pNS1, altered P9	(R135A)	This study
pNS912	pNS1, altered P9	(P208R)	This study
pNS913	pNS1, altered P9	(K17R)	This study
pNS914	pNS1, altered P9	(K17A)	This study
pNS915	pNS1, altered P9	(K17Q)	This study
pNS916	pNS1, altered P9	(Y72A)	This study
pNS917	pNS1, altered P9	(K125A)	This study
pNS918	pNS1, altered P9	(Q134E)	This study
pNS919	pNS1, altered P9	(F149V)	This study
pNS920	pNS1, altered P9	(F150A)	This study
pNS921	pNS1, altered P9	(F153A)	This study
pNS922	pNS1, altered P9	(Y74F)	This study
pNS923	pNS1, altered P9	(D103E)	This study
pNS924	pNS1, altered P9	(E104D)	This study
pNS926	pNS1, altered P9	(3 aa C-terminal deletion)	This study
pNS927	pNS1, altered P9	(7 aa C-terminal deletion)	This study
pNS96	pMG60+PRD1 gene *IX*		This study
pNS933	pNS96, altered P9	(R135K)	This study
pNS936	pNS96, altered P9	(P208R)	This study
pNS942	pNS96, altered P9	(K17Q)	This study
pNS945	pNS96, altered P9	(Y74F)	This study
pNS948	pNS96, altered P9	(K125A)	This study

Numbers refer to the PRD1 genome coordinates (M69077).
[a] Cm, chloramphenicol; Ap, ampicillin; superscript R, resistance.

taken at three hours post-induction, and treated similarly. The samples were then analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Plasmid pNS1 producing soluble wild-type P9 and vector alone were used as controls.

P9 mutant protein producing *E. coli* HMS174 (pLM2) cells with gene *IX* mutant constructs were infected with the P9 deficient amber mutant *sus1*[89] and scored for plaques. Cloned wild-type gene *IX* (pNS1) and the vector pSU18 alone were used as positive and negative controls, respectively. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 0.1 mM in the complementation analysis, except for plasmid pNS912 (coding for mutation P208R) for which also 0.4 mM and 1.0 mM concentrations were tested.

DNA packaging assay

For production of recombinant P9 extract, HMS174 (pNS96) cells were grown at 28 °C to a density of approximately 1×10^7 cfu/ml and production of P9 was induced with 100 μM IPTG. After three hours cells were collected by centrifugation and resuspended 1:100 (v/v) in 20 mM $NaHPO_4$ buffer (pH 7.2). For wild-type P9 extract, cells were disrupted by three passages through a French pressure cell (900 psi) and the debris was removed by ultracentrifugation (Beckmann Ti50 rotor, 20,000 rpm, one hour, 4 °C). The amount of P9 was estimated from SDS-PAGE using Coomassie brilliant blue staining. Aliquots of the supernatant were stored at −80 °C with 15% (v/v) glycerol. A freshly thawed aliquot of P9 extract was used for each set of packaging reactions. For analysis of the P9 mutant extracts, cells were produced and stored as described above, but the thawed cells were disrupted by sonication (two times ten seconds) and used directly for packaging reactions along with similarly produced wild-type P9 extract as a control. Production and solubility of P9 mutant proteins was assayed by SDS-PAGE and Coomassie brilliant blue staining.

PRD1-1 DNA, containing the *lacZ*α-insert, was isolated from purified virus particles by SDS-treatment, phenol-ether extraction and ethanol-precipitation as described[31] and its amount was estimated from an agarose gel using ethidium bromide staining and control DNA with known concentration. PRD1-1 DNA was stored in aliquots in 10 mM Tris (pH 8) with 5% glycerol at −20 °C.

Procapsids were produced by *sus1*-infection: DS88 cells were grown at 37 °C to a density of 1×10^9 cfu/ml and infected using a MOI of 8. After 40 minutes, the cells were collected by centrifugation and resuspended 1:100 (v/v) in 20 mM Tris (pH 7.2) and disrupted by sonication (two times ten seconds). Glycerol was added to a final concentration of 15% and aliquots of the procapsid extract were stored at −80 °C. A freshly thawed aliquot was used in each set of packaging reactions.

The standard DNA packaging reaction contained (in 200 μl): 25 μl of Sus1 procapsid extract, 15 μl of P9 extract, PRD1-1 DNA (5–10 μl, ∼250 ng) and 50 mM Tris buffer (pH 7.2), 3% PEG8000, 10 mM NaCl, 10 mM $MgCL_2$,

5 mM $CaCl_2$, 2.5 mM ATP. The components of the packaging reaction (except for extracts and DNA) were mixed in an Eppendorf tube, DNA, P9 and particle extracts were added, and the reaction was gently, but thoroughly mixed. The packaging mixture was incubated for 45 minutes at 37 °C, the reaction was stopped by addition of DNaseI to 25 μg/ml (five minutes incubation at 37 °C), and transferred onto ice. Aliquots were removed and plated either on *E. coli* DH5α, carrying the PRD1 receptor coding plasmid pJB15,[90] or DS88 cells. The top agar was supplemented with 0.4 mM IPTG and 0.33 mg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), to induce and detect the LacZα-complementation, respectively. The plates were incubated overnight at 37 °C. The effect of RNases was tested by adding one to ten units of RNaseA, RNaseI or RNaseIII to the reaction. One unit of apyrase was used to test the effect of NTP depletion. In addition to Tris buffer (pH 7.2 and 8.0), several other buffers (pH 6–8) were preliminarily tested when optimising the reaction.

Analytical methods

The Coomassie brilliant blue method using bovine serum albumin (BSA) as a standard[91] was used for determining the protein concentration of purified virus preparations. SDS-PAGE was performed as described.[92]

For Western blotting the proteins were transferred from SDS-PAGE (17% acrylamide) gels onto polyvinylidene difluoride (PVDF) membranes (Millipore). Polyclonal antiserum against PRD1 P9[35] was used as the primary antibody. Proteins were visualised using the Supersignal® West Pico Chemiluminescent Substrate (Pierce) with HRP-conjugated swine anti-rabbit IgGs (Dako).

Sequence analysis

Amino acid sequences were aligned with the AlignX® programme of the Vector NTI™ Suite 8 package (Informax Inc.) and further adjusted manually. PSI-Blast[64] was used for iterative protein database searches.

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